THE INHIBITORY PROPERTIES OF AND MODE OF ACTION OF PLANT ESSENTIAL OILS AND FRUIT EXTRACTS ON PROTOZOAN PARASITES

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ABSTRACT

The main aims and objectives of this study was to determine if plant essential oils (PEOs) and polyphenol-rich fruit extracts (PRFEs) could reduce the viability of *Giardia duodenalis* trophozoites, *Trypanosoma cruzi* epimastigotes and *Cryptospordium parvum* oocysts *in vitro*. All PEOs tested reduced epimastigote and trophozoite viability at a concentration of 0.02% v/v, with titrations of the PEOs showing a concentration dependant decrease in viability. The minimum inhibitory concentrations (MICs) of PEOs demonstrated that myrtle and elemi oil were the most active PEOs (trophozoites = 0.005% v/v; epimastigotes = 0.00125% v/v) with the terpenes, α-pinene and limonene, constituents of these oils, being responsible for their action. Incubation of palmarosa oil and its terpene, geraniol, with *C. parvum* oocysts caused the almost complete excystation of oocysts (in the presence of increased temperature and time), with geranium oil and its terpene, citronellol, being nearly as effective. PRFEs reduce trophozoite viability, with 4 members of the Rosaceae Family causing complete reduction at 167 μg ml^{-1}, possibly through their ellagitannin content. Cloudberry extract was found to have an MIC comparable to the drug metronidazole (67 μg ml^{-1}). The historical use of blueberries for the treatment of diarrhoeal diseases was demonstrated by the ability of blueberry PRFE, pressed juice and drink to kill trophozoites. Protein expression was both inhibited and upregulated in several proteins in whole cell lysates of PEO treated trophozoites, indicating a supplemental intracellular mode of action. Both PEOs and PRFEs cause morphological changes to epimastigotes and trophozoites through flagellar truncation and internalisation, swelling and rounding of the cell body, cytoplasmic condensation and the formation of large membrane protrusions. These indicate an action on the membrane itself with possible changes in osmoregulation. Both PEOs and PRFEs can be considered to be candidates for novel drug discovery for the treatments of cryptosporidiosis, giardiasis and American trypanosomiasis.

Key words: *Giardia duodenalis*; *Trypanosoma cruzi*; *Cryptosporidium parvum*; plant essential oils; monoterpenes; terpenic compounds; fruits; polyphenol-rich fruit extracts.

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I would like to finish with the following 2 thoughts:

"...it is a capital mistake to theorize before one has data. Insensibly one begins to twist facts to suit theories, instead of theories to suit fact" - Sir Arthur Conan Doyle.

"When you have eliminated the probable whatever remains, no matter how improbale, must be the truth." – Sherlock Holmes

ABBREVIATION LIST

1DE: 1-dimensional gel electrophoresis
2DE: 2-dimensional gel electrophoresis
°C: Degrees Centigrade
AD: *Anno Domini*
AIDS: Acquired immunodeficiency syndrome
Anon.: Anonymous
APS: Ammonium persulphate
BC: Before Christ
BB: Bouvrage Blaeberry drink
BGF: Bunch Grass Farm
BHK: Baby hamster kidney
BP: Before Present
Ca^{2+}: Calcium ions
CaCo2: Human epithelial colorectal adenocarcinoma cell line
CD4: Cluster of Differentiation type 4
CDC: Centre for disease control and prevention
CGE: Cyanidin-3-glucoside equivalents
CNS: Central nervous system
COWP: *Cryptosporidium* oocyst wall protein
CWP: Cyst wall proteins
DAPI: 4′6-diamidino-2-phenyl indole
DAT: Diallyl trisulphide
DIC: Differential interference contrast
DMSO: Dimethyl sulphoxide
DNA: Deoxyribonucleic acid
dNTPs: Deoxyribonucleotide triphosphates
DTT: dithiothreitol
ED_{50}: 50% Effective Dose (that quantity of drug required to produce a specified effect in 50% of the animal population)
EDTA: Ethylenediaminetetraacetic acid
EPA: Environmental Protection Agency
ER: Endoplasmic reticulum
ESV: Encystation-specific vesicle
EtOH: Ethanol
Euc.: Eucalyptol
FB: pressed Finnish Blueberries
FDA: Food and drug administration
FEMA: Flavour and Extract Manufacturer's Association
FFDCA: Federal Food, Drug and Cosmetic Act
FITC: Fluorescein isothiocyante conjugate
FITCmAb: Fluorescein isothiocyante conjugated monoclonal antibody
FK: Freeze killing
FW: Formula Weight
g: Gravities
G: *Giardia duodenalis* trophozoites
GAE: Gallic acid equivalents

Gal/GalNAc: D-galactose/N-acetyl-D-galactosamine
G-C FITCmAb: Fluorescein isothiocyante conjugated monoclonal antibody for *Giardia* cysts and *Cryptosporidium* oocysts
GC-MS: Gas chromotography – Mass spectroscopy
GIT: Gastrointestinal tract
GLC: Gas liquid chromatography
gPDI: *Giardia* protein-disulphide isomerase
GRAS: Generally Recognised as Safe
h: Hour
HAART: Highly active antiretroviral therapy
HBSS: Hanks balanced salt solution
HCl: Hydrochloric acid
HCT-8: Human colorectal adenocarcinoma cell line
HIV: Human immunodeficiency virus
HK: Heat killing
HL-60 cells: Human lymphocyte-60 cells
HMEM: Hanks minimal essential medium
HPLC: High performance liquid chromatography
hsp: Heat shock protein
[^{3}H] thymidine: Tritiated thymidine
HSV: Herpes simplex virus
IC_{50}: 50% Inhibitory Concentration (that quantity of drug required to inhibit 50% in the growth or viability of a population)
IC_{90}: 90% Inhibitory Concentration (that quantity of drug required to inhibit 90% in the growth or viability of a population)
ID_{50}: 50% Infectious Dose (dose required to cause infection in 50% of test subjects)
IgG: Immunoglobulin type G
I.M.: Intramuscular
IMS: Immunomagnetic separation
IOWA: Iowa isolate of *Cryptosporidium parvum* from BGF
I.P.: Intraperitoneal
I.V.: Intravenous
kDa: Kilo Daltons
k-DNA: Kinetoplast DNA
kg: Kilogram
kg^{-1}: Per kilogram
LB: Lysis buffer
LD_{50}: 50% Lethal Dose (the dose which kills 50% of the animals tested)
Lim.: Limonene
µg: Microgram
µl: Microlitre
µm: Micrometre
µM: Micromolar
MD: Isolate of *Cryptosporidium parvum* from MRI
Metro: Metronidazole
MIC: minimum inhibitory concentration
min: Minute
ml: Millilitre

ml^{-1}: Per millilitre
$MgCl_2$: Magnesium chloride
mg/kg/day: Milligrams per kilogram per day
MLC: minimum lethal concentration
mm: Millimetre
MPC-6: magnetic particle concentrator-6
MRI: Moredun Research Institute
MRSA ref lab: Methicillin Resistant *Staphylococcus aureas* reference laboratory
MTS: 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MW: Molecular Weight (marker)
NaOH: Sodium hydroxide
nm: Nanometres
OM: Outer membrane
Optimem: Modified Eagle's Minimum Essential Medium
PC: Phosphatidyl choline
PCR: Polymerase chain reaction
PEO: Plant essential oil
PES: Phenazine ethosulphate
PFOR: pyruvate:ferredoxin oxidoreductase
PGE_2: prostaglandin E_2
Pin.: α-pinene
PRBE: Polyphenol-rich blueberry extract
PRFE: Polyphenol-rich fruit extract
PV: Peripheral vesicle
PVP: Polyvinylpyrrolidone
QMU: Queen Margaret University
RFLP: Restriction fragment length polymorphism
RNA: Ribonucleic acid
rRNA: Ribosomal Ribonucleic acid
RPMI 1640: Basal media
RT: Room temperature
SCRI: Scottish Crop Research Institute
SDS-PAGE: Sodium dodecylsulphate – Polymerised acrylamide gel electrophoresis
Sec: Seconds
SEM: Scanning electron microscopy
SHARP: Soutra Hospital Archaeoethnopharmacological Research Project
SI: Selectivity index
Sp.: Sporozoites
SPDL: Scottish Parasite Diagnostic Laboratory
TAE buffer: Tris-acetate-EDTA buffer
TBMM: Trypan blue, motility and morphological assay
TEM: Tunnelling electron microscopy
TEMED: N,N,N', N'-Tetramethylethylenediamine
TRAP-C1: Thrombospondin-related adhesive protein 1
TTO: Tea tree oil
UK: United Kingdom

USA: United States of America
UV: Ultra violet
V: Volts
v/v: volume to volume
w/v: weight to volume
$Well^{-1}$: Per well
WHO: World Health Organisation

FIGURE AND TABLE LIST

CHAPTER 3 – *Cryptosporidium parvum*

CHAPTER 4 – *Trypanosoma cruzi*

CHAPTER 5

CHAPTER 6

CHAPTER 7

CONTENTS

CHAPTER 1

Introduction

Plants and their extracts have been used for many centuries as treatments for various ailments from headaches to parasite infections (Jones 1996), yet it has only been in the last 20 – 30 years or so that scientists have seriously begun to look closely at these plants and discover if these traditional remedies truly work and if so, how they work.

Less than 10% of approximately 250,000 of the world's flowering plant species have been tested and examined scientifically for their medicinal properties (Editorial 1994). This means that there is a vast, untapped reservoir of potential new drugs available to study. Furthermore, several parasites, such as *Giardia duodenalis* (Upcroft, Upcroft and Boreham 1990; Upcroft & Upcroft 2001), *Plasmodium falciparum* (Le Bras & Durand 2003; Sibley & Hunt 2003; Wilairatana *et al*. 2002), *Trypanosoma cruzi* (Camandaroba *et al*. 2003; Filardi & Brener 1982; Sgambatti de Andrade *et al*. 1996) and *Leishmania donovani* (Sundar 2001; Sundar *et al*. 2001) are becoming more resistant to conventional drugs, or in the case of *Cryptosporidium* spp. lack effective chemotherapeutics (Mead 2002; Smith & Corcoran 2004) and new therapies, therefore require to be found.

1:1 Historical use of plants

Most of the knowledge about the therapeutic use of plants has been through folklore and has been handed down by word of mouth. Other ways in which modern scientists can obtain such information is by examining ancient texts, such as the ancient Greek Dioscorides' *Materia Medica* or the medieval Macer Floridus' *De Viribus Herbarum* or Chinese herbalism. It was through this later route that a new pharmaceutical product was developed from the compound artemesinin, which when used in combination with modern synthetic chloroquine-derived compounds was found to be effective in the treatment of *P. falciparum* malaria (Simpson 2002). Artemesinin comes from the herb plant *Artemesia annua* (Figure 1.1) where use has been recorded since 341BC as a Chinese herbal remedy for malaria (Li & Wu 1998; O'Neill *et al*. 2001). Since the isolation of the antimalarial principle in 1970s (Li & Wu 1998) it has taken almost 30 years for it to become part of the World Health Organisation's Essential Medicines list, especially in cases where the parasite has

become resistant to both chloroquine and sulphoxine-pyramethamine (Simpson 2002).

Figure 1.1 Sweet Wormwood (*Artemesia annua*) and its active constituent artemisinin.
Illustration: Köhler's Medizinal-Pflanzen in naturgetreuen Abbildungen mit kurz erläuterndem Texte: Atlas zur Pharmacopoea germanica (Köhler's Medicinal Plants) 1887. published in four volumes. Franz Eugen Köhler and ed. Gustav Pabst. Photograph: J-P Anthony (copyright© Scottish Parasite Diagnostic Laboratory [SPDL], Glasgow and Queen Margaret University [QMU], Edinburgh) photographed at the Botanic Gardens, Glasgow, 2004.

Sometimes the simple aqueous infusion/decoction of the plant material can be used to great effect. The term decoction refers to the process where plant material (usually the roots or bark) is added to water and then boiled, allowed to cool and then given to drink, whereas an infusion is usually made when the leaves and flowering tops (either dried or fresh) are added to boiled water and allowed to infuse like a tea. In the case of malaria, a recent study using a local remedy of N'Dribala (*Cochlospermum planchonii*) roots in a decoction was shown to be effective, resulting in 52% of the patients reaching parasitological cure (c.f. 57% in chloroquine treated patients) in the treatment of uncomplicated *P. falciparum* infection in humans and was comparable to treatment with Chloroquine, but with no significant side effects (Benoit-Vical *et al*. 2003).

Many of the studies being undertaken currently involve investigating the use of aqueous infusions or various alcoholic extracts. However, it is likely that purified plant essential oils may be a particularly good way of preventing and treating

diseases caused by parasites. Properties such as low density (around 0.94 g ml^{-1}) and ready diffusion across cell membranes (due to membrane lipid solubility) may enhance the targeting of active constituents within the oil to intracellular parasites (Boyom *et al*. 2003). The oils and their constituents have been well characterised by high performance liquid chromatography (HPLC) and the suppliers of these oils have good quality control in regard to consistent composition and purity, due to the requirements of the food and aromatherapy industries. As the oils lack contaminants and because they are 'clean', then it is easy to separate the compounds within the oils on HPLC or gas liquid chromatography (GLC). A major advantage of using oils, especially those which are commercially sourced, is that it is possible to get the same batch from suppliers over long periods of time. It is now possible to make synthetic oils, with many of the constituents being characterised and synthesised in the laboratory.

Although the greatest amount of research concerning the effects of plants on parasite infections has been carried using aqueous or alcoholic extractions some, work has been undertaken using plant oils.

1:2 Antiparasitic effects of plant essential oils (PEOs)

Generalised parasiticidal actions

Of the plant essential oils available and currently used only one has been investigated for actions against multiple prokaryotic or eukaryotic organisms. Garlic oil has a multitude of effects including antibacterial, antifungal, antiviral and antiparasitic (Ankri & Mirelman 1999). As a parasiticidal compound, it affects the growth of over 12 different parasites by cysteine proteinase inhibition and parasite proliferation by the inhibition of phosphatidyl choline (PC) synthesis. The allicin constituent of garlic (Figure 1.2) inhibits the growth of *Entamoeba histolytica* at concentrations of 30 µg ml^{-1} *in vitro* (Ankri & Mirelman 1999; Mirelman, Monheit and Varon 1987) with lower concentrations able to inhibit the virulence of trophozoites by 90% as measured by the ability to destroy baby hamster kidney (BHK) cell monolayers *in vitro* (Ankri *et al*. 1997). This and other garlic oil constituents inhibit the growth of other protozoan parasites including: *G. lamblia* (syn. *intestinalis* and *duodenalis*),

Leptomonas colosoma, Crithidia fasculata (Ankri & Mirelman 1999; Lun *et al*. 1994), *Cryptosporidium baileyi* (Sreter, Szell and Varga 1999), *Tetratrichomonas gallinarum, Histomonas meleagridis* (Zenner *et al*. 2003), *G. intestinalis* (Harris *et al*. 2000), *P. berghei* (Perez, De la Rosa and Apitz 1994), *T. brucei brucei, T. b. rhodesiense, T. b. gambiense, T. evansi, T. congolense, T. equiperdum* (Lun *et al*. 1994) and *T. cruzi* (Urbina *et al*. 1993).

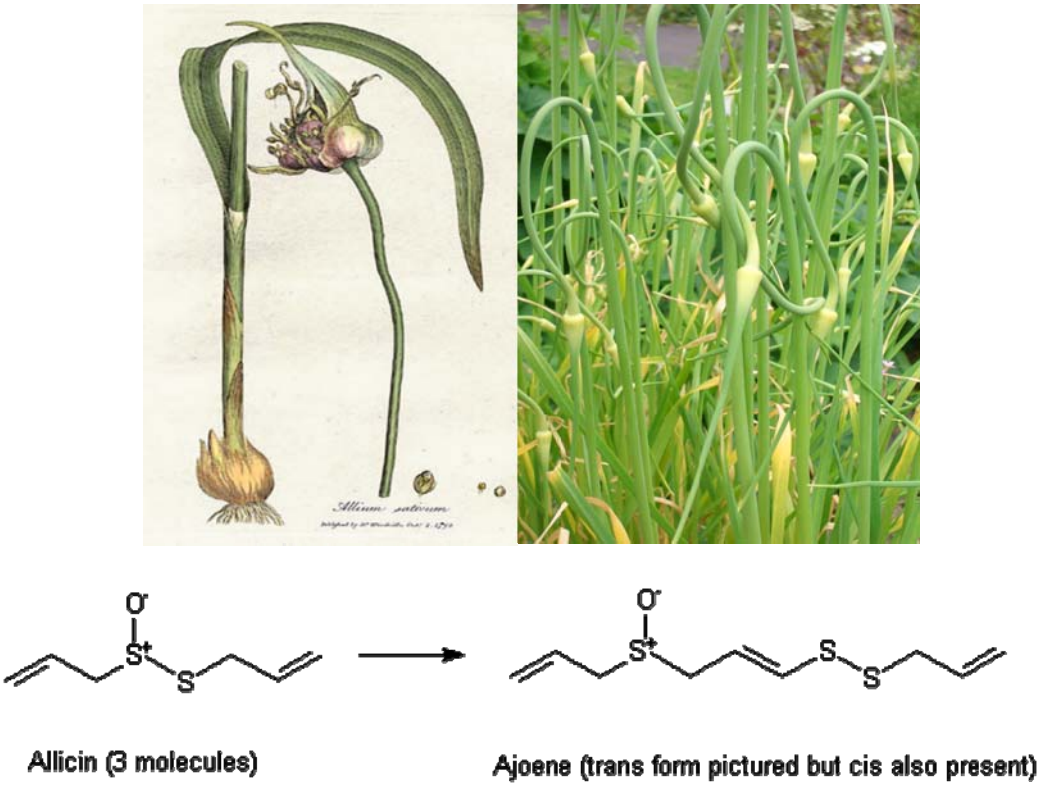


Figure 1.2 Garlic (*Allium sativum*) and its main constituent allicin with its condensation product ajoene.
Illustration: Medical botany, William Woodville. Published by James Phillips, London, 1793, Vol. 3. Photograph: J-P Anthony (copyright© SPDL and QMU) photographed at the Botanic Gardens, Glasgow, 2004.

The mode of action of allicin and its condensation product, ajoene, have both been investigated. Reported mechanisms of action include interaction with important thiol-containing enzymes (Ankri & Mirelman 1999). The specific enzymes vary from organism to organism but in the case of *E. histolytica*, allicin inhibits cysteine proteinases, alcohol dehydrogenases (Ankri *et al*. 1997) and thioredoxin reductases (Ankri & Mirelman 1999). Ajoene can inhibit *T. cruzi* proliferation by possible

inhibition of PC biosynthesis (Urbina *et al*. 1993). Allicin was also found to be toxic to mammalian cells but at a concentration of greater than 100 μg ml^{-1} (c.f. 5 μg ml^{-1} for *E. histolytica*) (Ankri *et al*. 1997). This difference may be explained by mammalian cells having a much higher level of glutathione than microbial cells allowing the cell to reactivate enzymes inhibited by allicin (Rabinkov *et al*. 1998). The modes of action of these constituents of garlic oil have only been investigated in *E. histolytica* and *T. cruzi*, but they may act in similar ways in other parasites.

Of the oils shown in Table 1.1, oregano and basil have been shown to be effective against more than 2 parasites. In human trials, the emulsified oil of oregano was given orally to volunteers for 6 weeks which resulted in the clearance of *E. hartmanni, Endolimax nana and Blastocystis hominis* (Force, Sparks and Ronzio 2000). When given in the feed of broiler chickens infected with *Eimeria tenella*, oil of oregano exerted an anticoccidial effect although this was lower than that of the favoured drug, Lasalocid®.

Treatment of head lice (*Pediculus humanus capitis*) with oregano oil as a 1% solution diluted in water produced 100% mortality in adults, when followed by a rinse mixture of the oil (0.1%), malt vinegar (49.5%) and water (49.5%) 17 h later and a 99.3% mortality for eggs (Veal 1996). The adult mortality rate was found to be the same for thyme oil (100%) but with a much lower activity against eggs (50.8% mortality). Essential oil isolated from thyme has also been found to inhibit the growth *in vitro* of *T. b. brucei* at a level comparable to the drug Suramin®. The 50% Effective Dose (ED_{50}, that quantity of oil required to produce a specified effect in 50% of the animal population) was 0.4 μg ml^{-1} for thyme oil and 0.5 μg ml^{-1} for Suramin® (Mikus *et al.* 2000). When thyme oil was tested *in vitro* with *G. lamblia* cysts, it's effect following a 60 min exposure, was comparable to the drug metronidazole (lethal effect = thyme oil 91.1%; metronidazole 89.4%) (Sahebani, Farsangi and Movahed 2004).

Basil oil inhibits the growth of *T. cruzi* epimastigotes *in vitro* with a 50% Inhibitory Concentration (IC_{50}, that quantity of oil required to inhibit 50% in the growth or

viability of a population) determined to be 102 μg ml^{-1} and lysis of trypomastigotes at an IC_{50} of 467.5 μg ml^{-1}. Following treatment with basil, epimastigotes examined under scanning electron microscopy (SEM) showed no apparent changes to the plasma membrane but morphological changes to the cell body was exhibited with basil treated cells being rounded when compared to untreated controls (Santoro *et al*. 2007b). This would indicate that basil does not exert an effect via direct interaction with components of the plasma membrane but with the intracellular organelles. This oil and its major constituent, eugenol, were also found to inhibit *H. contortus* ova from hatching *in vitro* (Pessoa *et al*. 2002). When given orally to mice infected with *P. berghei*, basil oil suppressed parasitaemia by up to 77.8 % at 500 mg/kg/day, although this was far lower than that obtained with chloroquine given at a dose of 10 mg/kg/day (100 % suppression; Tchoumbougnang *et al*. 2005).

In vivo experiments in chickens have shown that both cinnamon and lemon oil have anti-flagellate activity against *T. gallinarum* and *H. meleagridis* (Zenner *et al*. 2003) (Table 1.1 and 1.3). The effective minimal lethal concentration of cinnamon oil used on both parasite species was determined to be 0.25 μl ml^{-1} and 0.5 μl ml^{-1} respectively, with that of lemon oil being 0.125 μl ml^{-1} and 1 μl ml^{-1} respectively (Zenner *et al*. 2003). In addition to this anti-flagellate activity, cinnamon also has insecticidal actions with 10 mg g^{-1} of the essential oil impregnated filter paper, fed to termites (*Coptotermes formosanus*), killing them all within 7 days (Chang & Cheng 2002). The active constituent of the oil, cinnamaldehyde, was more effective at lower concentrations (1 mg g^{-1}) than the oil (Chang & Cheng 2002). Cinnamon oil when used as a treatment for *P. humanus capitis* in the manner described for oregano and thyme, also caused louse mortality but was not as effective (adults: 86%, eggs: 25.7%) although 100% mortality rates were achieved when ethanol was used as a solvent in the pre-rinse mixture. The concentration of ethanol used did not to contribute to the lethality of cinnamon oil and that the phenolic compounds within the oil may be responsible for the lousicidal actions exhibited, possibly through neurotoxicity (Veal 1996).

Lemon balm, also known as balmint, has been tested *in vitro* against *T. brucei* and *L. major* both of which have conventional treatments available but are currently expensive or have strong side effects. The essential oil was found to inhibit the growth of *T. brucei* (ED_{50} 3.9 μg ml^{-1}; Suramin ED_{50} 0.5 μg ml^{-1}) and *L. major* (ED_{50} 7 μg ml^{-1}; amphotericin B ED_{50} 0.3 μg ml^{-1}) (Mikus *et al*. 2000). When tea tree oil (TTO) was used in these experiments the results for *T. brucei* inhibition indicated that this oil might be an alternative to Suramin® for the treatment of trypanosomiasis (ED_{50} 0.5 μg ml^{-1} for both compounds). One of the major constituents of TTO is terpinen-4-ol which has a greater inhibitory action on the blood stream forms of *T. brucei* (ED_{50} 0.02 μg ml^{-1}) and was over a 1000 fold more toxic to the parasite than the human cell line HL-60 (Mikus *et al*. 2000). It was proposed that a deeper investigation of these compounds trypanocidal activity should be undertaken to determine their effectiveness as a new drug therapy.

Antitrypanosomal activity of PEOs

Again garlic appears to be the most active compound, with the constituent allicin being of particular importance (Table 1.1). The chemically stable transformation product of allicin is diallyl trisulphide (DAT) and is effective in inhibiting the growth of the many species of the bloodstream form of trypanosomes, especially those responsible for African trypanosomiasis (Lun *et al*. 1994). These include *T. brucei brucei, T. b. rhodesiense, T. b. gambiense, T. evansi, T. congolense and T. equiperdum* with DAT inhibiting growth at concentrations comparable to the commercial drug Suramin® (trypanosome IC_{50} = 0.8 - 5.5 μg ml^{-1} (4.5 – 31 μM); Suramin® IC_{50} = 10 μM; Lun *et al*. 1994). This compound has been reported to have been used in China for the treatment of many diseases, such as bacterial and fungal infections, with only mild side effects (Lun *et al*. 1994). A condensation product of allicin is effective against both the epimastigote amastigote forms of *T. cruzi* to an even greater degree. Ajoene immediately inhibits the proliferation of epimastigotes at a concentration of 80 μM, reducing their proliferation by 50% with 40 μM and causing cell lysis within 24 h with 100 μM (Urbina *et al*. 1993). Concentrations as low as 40 μM were enough to completely eliminate *T. cruzi* amastigotes from Vero cells *in vitro* within 96 h. The inhibition of epimastigote growth was accompanied by

changes in phospholipid composition of the parasite cell membrane, causing the previously most abundant phospholipids (PC) to become the least abundant and their immediate precursor (phosphatidylethanolamine) becoming the most abundant. This suggests that ajoene exerts its effects by inhibiting the final stage of PC biosynthesis and alters the phospholipid composition of the cell. This was observed ultra structurally with a concentration dependent alteration in intracellular membranous structures (Urbina *et al*. 1993). Garlic with its trypanocidal actions may yet be used as an alternative treatment for trypanosomiasis, especially African trypanosomiasis, considering that the drugs currently in use for the later stages of this disease are extremely toxic (Sibley & Hunt 2003).

The essential oils of lemon balm, peppermint, thyme, TTO and the African plant *Strychnos spinosa* have also been tested *in vitro* for activity against *T. brucei* with varying degrees of success (Table 1.1). Of these oils, TTO has the most trypanocidal activity followed by thyme, peppermint and finally lemon balm (Mikus *et al*. 2000). All the oils tested showed a much greater toxicity for the parasite than for the mammalian cell suggesting that these oils could be a source of novel drugs which could be directed specifically against the parasite. This was also shown in the same series of experiments when the terpene compounds of these oils were also tested. In this case only terpinen-4-ol from TTO had a relatively high trypanocidal activity with a selectivity of 1000, which is within the range of commercially available drugs such as Suramin® (Mikus *et al*. 2000).

The leaves of *Strychnos spinosa* are used in African traditional medicine for the treatment of African trypanosomiasis. An essential oil and its constituents have been tested against the bloodstream form of *T. brucei in vitro* and also against the murine J774 macrophage (Hoet *et al*. 2006). The oil itself, whilst effective against the parasite (IC_{50} = 13.5 μg ml^{-1}) was sadly found not to be selective for the parasite alone (selectivity index (SI) = 4.4). However, constituents of the oil (E-nerolidol and linalool) were found not only to have a more potent effect on the parasite but also a more selective one (IC_{50} = 1.7 and 2.5 μg ml^{-1}; SI = 35.7 and > 40 respectively).

These 2 terpenes show promise as novel drug candidates and require further *in vitro* and *in vivo* testing.

Thyme essential oil and its major constituent thymol have also been more recently investigated for effects on both the insect and bloodstream forms of *T. cruzi* (Table 1.1). Both were found to inhibit the growth of epimastigotes after 24 h incubation *in vitro* (Thyme oil IC_{50} = 77 μg ml^{-1}; Thymol IC_{50} = 62 μg ml^{-1}) and also had higher trypanocidal effects with trypomastigotes over the same incubation period (Thyme oil IC_{50} = 38 μg ml^{-1}; Thymol IC_{50} = 53 μg ml^{-1}). Although morphological analysis of the parasites SEM showed little alteration at the plasma membrane, transmission electron microscopy (TEM) showed cytoplasmic swelling with occasional morphological alteration in both the plasma and flagellar membrane (Santoro *et al*. 2007a). In addition the oils of basil, clove, lemon grass, oregano and yarrow have also been tested against *T. cruzi* epimastigotes and trypomastigotes and exhibit a range of IC_{50} from 77 – 145.5 μg ml^{-1} for epimastigotes and 15.5 – 467.5 μg ml^{-1} for trypomastigotes (Santoro *et al*. 2007a, 2007b and 2007c).

Anticoccidial and antiflagellate activity of PEOs

Again garlic has been shown to have important parasiticidal actions (Table 1.1), with activity against the coccidian parasite *C. baileyi* (Sreter, Szell and Varga 1999), the poultry flagellates *T. gallinarum* and *H. meleagridis* (Zenner *et al*. 2003) *and* the human pathogenic flagellate *G. intestinalis* (Harris *et al*. 2000; Lun *et al*. 1994) being observed. When tested in chickens, an extract of garlic was found to be only partially effective with a 24.4% reduction in *Cryptosporidium* oocyst output. The 2 commercially available derivatives of the anticoccidial drug triazinone fared no better. It was concluded that none of the drugs could be recommended for chemoprophylaxis or therapy of cryptosporidiosis in chickens (Sreter, Szell and Varga 1999). Garlic's antiflagellate activity, however, is far more encouraging. In *G. intestinalis*, both whole garlic extract and its constituents have definite antigiardial effects, causing a loss of flagellar movement and motility, loss of osmoregularity, disc fragmentation and flagellar internalisation (Harris *et al*. 2000) and growth inhibition (Lun *et al*. 1994). Although *T. gallinarum* and *H. meleagridis* are both

killed by garlic (Zenner *et al*. 2003) the mechanism of action has yet to be determined. The same effect is also observed with both the oils of Cinnamon and Lemon (Zenner *et al*. 2003). When oil of oregano was given in their feed to chickens experimentally infected with the coccidian parasite *Eimeria tenella* excretion of oocysts was reduced and an increase in survival was observed (Giannenas *et al*. 2003). Although these effects were less than lasalocid, it may be that this oil can be a source of novel anticoccidial drugs.

1:3 Toxicity of PEOs

As with any chemotherapeutic compound, there can be side effects or toxicity, usually at concentrations greater than therapeutic doses. Generally plant oils are well tolerated in humans and animals (Tisserand & Balacs 1995), although there can be differences in this tolerance between species. However, although most oils are well tolerated with only mild side effects, some are not recommended for internal or external use such as the oil from the bark of cinnamon and lemon oil for external use as it is known to be phototoxic (Tisserand & Balacs 1995). A summary of toxic oils can be seen in Table 1.2.

Cytotoxicity

Many of the oils that affect parasites also exhibit either *in vivo* or *in vitro* toxicity to varying degrees. This toxicity can be mild or the selectivity of the oil is such that the parasite is affected to a greater degree than the host. For example the active constituents of garlic have wide ranging antiparasitic effects (Ankri *et al*. 1997; Ankri & Mirelman 1999; Harris *et al*. 2000; Lun *et al*. 1994; Mirelman, Monheit and Varon 1987; Perez, De la Rosa and Apitz 1994; Urbina *et al*. 1993; Zenner *et al*. 2003), can be both cytotoxic and genotoxic *in vitro* (Musk, Clapham and Johnson 1997) and are lethal if given intragastrically to rats causing pulmonary oedema (Joseph, Rao and Sundaresh 1989). However, if the oil of garlic is given orally to rats at the same concentrations, it is not toxic (Joseph, Rao and Sundaresh 1989).

Table 1.1 Plant oils with known action against parasites.

FAMILY	BOTANICAL NAME	VULGAR NAME	PARASITE(S) AFFECTED
Alliaceae	*Allium sativum*	Garlic	*G. duodenalis* (syn. *intestinalis* and *lamblia*), *L. major, L. colosoma, C. fasculata, Cryptosporidium* spp., *T. gallinarum, H. meleagridis, P. berghei, T. brucei brucei, T. b. rhodesiense, T. b. gambiense, T. evansi, T. congolense, T. equiperdum, T. cruzi, E. histolytica*
Annonaceae	*Hexalobus crispiflorus*	?	*P. falciparum*
	Pachypodanthium confine	Bohingo	*P. falciparum*
	Xylopia aethiopica	African Guinea Pepper	*P. falciparum*
	Xylopia phloidora	Bolando	*P. falciparum*
Asteraceae	*Achillea millefolium*	Yarrow	*T. cruzi*
Chenopodiaceae	*Chenopodium ambrosioides*	Epazote / Jesuit's Tea	*L. amazonensis*
Cochlospermaceae	*Cochlospermum planchonii*	False Cotton / N'Dribala	*P. falciparum*
Euphorbiaceae	*Antidesma laciniatum*	?	*P. falciparum*
	Croton cajucara	Sacaca	*L. amazonensis*
Lamiaceae	*Melissa officinalis*	Lemon Balm	*T. brucei*, *L. major*
	Ocimum gratissimum	Wild / African Basil	*P. berghei, H. contortus, T. cruzi C. elegans*
	Ocimum sanctum	Sacred	*E. hartmanni, E. nana, B. hominis, P. humanus capitis, E. tenella, T. cruzi*
	Oreganum vulgare	Balm/Basil Oregano	
	Tetradenia riparia	Nutmeg Bush	*P. falciparum*
	Thymus vulgaris	Thyme	*P. humanus capitis, T. cruzi, G. duodenalis*
Lauraceae	*Cinnamomum zeylanicum*	Cinnamon	*T. gallinarum* and *H. meleagridis*
Loganiaceae	*Strychnos spinosa*	Spiny Monkey Orange	*T. brucei*
Myristiceae	*Virola surinamensis*	Light Virola	*P. falciparum*
Myrtaceae	*Melaleuca alternifolia*	Tea Tree	*T. brucei*
	Syzygium aromaticum	Clove	*T. cruzi*
Poaceae	*Cymbopogon citratus*	Lemon Grass	*P. berghei, T. cruzi*
Ranunculaceae	*Nigella sativa*	Black Seed/Cumin	*Schistosoma mansoni*
Rutaceae	*Citrus limon*	Lemon	*T. gallinarum* and *H. meleagridis*

? = Name unknown

Table 1.2 Plant oils that demonstrate cytotoxic activity.

FAMILY	BOTANICAL NAME	VULGAR NAME	REF.
Alliaceae	*Allium sativum*	Garlic	(Joseph, Rao and Sundaresh 1989; Musk, Clapham and Johnson 1997)
Annonaceae	*Pachypodanthium confine*	Bohingo	(Boyom *et al*. 2003)
	Xylopia aethiopica	African Guinea Pepper	(Boyom *et al*. 2003; Gonzalez-Coloma *et al*. 2002; Nakanishi *et al*. 2003; Zeng *et al*. 2003)
Cochlospermaceae	*Cochlospermum planchonii*	False Cotton/ N'Dribala	(Benoit-Vical *et al*. 1999; Benoit-Vical *et al*. 2003; Vonthron-Senecheau *et al*. 2003)
Euphorbiaceae	*Antidesma laciniatum*	?	(Boyom *et al*. 2003)
	Croton cajucara	Sacaca	(Anazetti *et al*. 2003; Freire *et al*. 2003; Rodriguez & Haun 1999; Agner *et al*. 2001 and 1999)
Lamiaceae	*Melissa officinalis*	Lemon Balm	(Ballard *et al*. 2002; Mikus *et al*. 2000)
	Menthe piperita	Peppermint	(Gaworski *et al*. 1994; Lazutka *et al*. 2001; Nair 2001; Spindler & Madsen 1992; Vo, Chan and King 2003)
	Thymus vulgaris	Thyme	(Haroun, Mahmoud and Adam 2002; Wang *et al*. 1999)
Myriceae	*Eucalyptus globulus*	Eucalyptus	(Darben, Cominos and Lee 1998; De Vincenzi *et al*. 2002; Gouin & Patel 1996; Patel & Wiggins 1980; Schnitzler, Schon and Reichling 2001)
Myristicaceae	*Myristica fragrans*	Nutmeg	(Banerjee *et al*. 1994; Hallstrom & Thuvander 1997; Janssens *et al*. 1990; Pecevski *et al*. 1981; Sangalli & Chiang 2000)
Myrtaceae	*Eugenia caryophyleta*	Clove	(Abdo *et al*. 2001; Simpson 1998)
	Melaleuca alternifolia	Tea Tree	(Del Beccaro 1995; Jacobs & Hornfeldt 1994; Schnitzler, Schon and Reichling 2001; Soderberg, Johansson and Gref 1996)
Ranunculaceae	*Nigella sativa*	Black Seed/ Cumin	(Mansour *et al*. 2001; Worthen, Ghosheh and Crooks 1998; Zaoui *et al*. 2002; Ali & Blunden 2003)
Zingiberaceae	*Curcuma longa*	Turmeric	(Chan, Chi-Tang and Hsing 1995; Deshpande *et al*. 1998; Kandarkar *et al*. 1998)

? = Name unknown.

Similar findings occurred when testing the N'Dribala plant. A decoction of roots given to human volunteers with uncomplicated *P. falciparum* infections caused no significant side effects (Benoit-Vical *et al*. 2003) but, *in vitro*, the essential oil was cytotoxic to K562 cells (a human erythroblastic cell line) IC_{50} = 860 – 950 μg ml^{-1} (Benoit-Vical *et al*. 2003). This was, however, far lower than the reported IC_{50} value of the oil against the parasite (22 – 35 μg ml^{-1}) (Benoit-Vical *et al*. 2003).

Eucalyptus oil, sometimes known as camphor oil and its major constituent eucalyptol has been given "Generally Recognised as Safe" status by the Flavour and Extract Manufacturer's Association (FEMA) and is approved by the FDA for food additive use (De Vincenzi *et al*. 2002). However, the oil is also widely regarded as being extremely toxic if ingested, causing central nervous system (CNS) depression (fatigue, loss of consciousness, headaches, decreased alertness) and tonic-clonic seizures rapidly following its ingestion (Gouin & Patel 1996; Patel & Wiggins 1980). Toxicological data for humans show a minimum lethal dose of 50 mg kg^{-1} which is 500 times greater than the proposed total daily intake limit of 0.1 mg kg^{-1} eucalyptol (De Vincenzi *et al*. 2002). This oil may also be useful as an antiviral being 3 times more toxic to herpes simplex virus (HSV) types 1 and 2 than to RC-37 cells (African green monkey kidney cells) (Schnitzler, Schon and Reichling 2001).

Of the plant essential oils commonly used, TTO is probably the best known. It has been tested extensively by scientists for its antibacterial and antifungal effects with work now being carried out on its potential as an antiparasitic and antiviral agent. This oil is considered to be non-poisonous and as such is freely available 'over the counter' in chemists as an herbal remedy (Soderberg, Johansson and Gref 1996). TTO has not been shown to be cytotoxic *in vitro* to human epithelial and fibroblast cells (Soderberg, Johansson and Gref 1996). Only at concentrations greater than 100 μg ml^{-1} did cell viability rapidly decline, which was thought to be due to its lipophilic character, with the oil interacting with cellular membranes, disrupting normal membrane activity (Soderberg, Johansson and Gref 1996). Its use internally however, should be avoided as the ingestion of less than 5 ml by children under the age of 24 months has caused severe poisoning with ataxia and a drowsiness with difficulty to

arouse, until the stomach was cleared of oil (Del Beccaro 1995; Jacobs & Hornfeldt 1994).

During the course of investigating antiplasmodial actions of various Cameroonian essential oils (*Pachypodanthium confine*, *Antidesma laciniatum*, *Xylopia aethiopica*, *X. phloiodora* and *Hexalobus crispiflorus*) *in vitro* all were found to be effective against *P. falciparum*, however, 2 plants were found to have high IC_{50} values indicating their toxicity to erythrocytes (Boyom *et al*. 2003). These oils were from *P. confine* and *A. laciniatum*. An essential oil that showed similar properties was African Guinea Pepper (*X. aethiopica*) and the major constituent of this oil, acetogenin, which is common to the Annonaceae family of plants, has been tested for its anti-tumour activities. The acetogenins and their analogues have shown remarkable selective cytotoxicity against human tumour cells (Nakanishi *et al*. 2003; Zeng *et al*. 2003). Some of the acetogenins have actions that have been characterised to be mitochondrial complex I inhibitors having cytotoxic effects on a panel of tumour cell lines, including the multidrug resistant cell SW480, selective cytotoxicity to insect Sf9 cells (Fall armyworm [*Spodoptera frugiperda*] pupal ovarian cells) and also antitrypanosomal action (Gonzalez-Coloma *et al*. 2002). Other acetogenins have had their cytotoxicity to human cancer cell lines evaluated as being DNA topoisomerase I poisons (Lopez-Lazaro *et al*. 2001).

The diterpene compounds from *Croton cajucara* (Sacaca) such as dehydrocrotonin and its analogues exhibit potent antineoplastic properties in that they induce terminal differentiation in the target cancerous cell and, hopefully, trigger apoptosis. In one test system, dimethylamide-crotonin and its parent compound dehydrocrotonin inhibited the growth of leukaemic HL60 cells *in vitro* without causing serious damage to human peripheral blood mononuclear cells (Anazetti *et al*. 2003). In further tests using the same cells, the cytotoxic reaction was explained to be due to adduct formation with DNA and proteins and/or oxidative stress induction (Freire *et al*. 2003).

Lemon balm has been examined for both its antiparasitic effects and its cytotoxic effects on HL60 cells. When tested it was found to have a 3.6 – 6.5 fold greater selectivity for *T. brucei* and *L. major* than to HL60 as evaluated using the Alamar blue assay (Mikus *et al*. 2000). The oil was also shown to be without any significant side effects when used to treat agitation in severe dementia (Ballard *et al*. 2002).

Thyme, commonly used in the kitchen, when given to rats in their diet, was found to have no toxic effects even when 10% of their diet constituted of thyme leaves (Haroun, Mahmoud and Adam 2002). However, the acetophenone glycosides isolated from a butanol soluble fraction of thyme extracts have shown weak cytotoxicity to human leukaemic cells *in vitro*, the mode of action being through DNA synthesis inhibition (Haroun, Mahmoud and Adam 2002; Wang *et al*. 1999).

Not all essential oils appear to have such adverse effects. Some will be protective such the oil of black seed / black cumin (*Nigella sativa*) and its constituents. This oil has been shown to be hepatoprotective where liver injury was induced in mice by carbon tetrachloride poisoning (Mansour *et al*. 2001). High LD_{50} values (the dose which kills 50% of the animals tested) in rats and mice given the oil acutely and chronically suggest a low toxicity of the oil, although chronic administration can significantly increase haematocrit and haemoglobin levels in the rat as well as lowering its leukocyte and platelet count (Zaoui *et al*. 2002). In general, this oil has hepatoprotective effects and can help protect against the chromosomal aberrations induced as a result of *Schistosoma mansoni* infection (Aboul-Ela 2002) and may be protective in this disease by modulating the immune response and reducing inflammation (Mahmoud, El-Abhar and Saleh 2002). Some possible protective effects of nutmeg oil are its ability to affect enzymes associated with activation and detoxication of chemical carcinogens and mutagens. When gavaged in mice over 14 days, oil of nutmeg increased levels of cytochrome P450 significantly and aryl hydrocarbon hydroxylase activity was significantly reduced. Enzyme activities that were also elevated by this oil include glutathione S-transferase and acid-soluble sulphydryl (Banerjee *et al*. 1994).

Dermatological reactions

Many plant oils are used safely in aromatherapy where they are often in contact with the skin, although few oils have actually been tested thoroughly for dermatological reactions, with dermatitis or other allergic reactions usually reported in the worst instances. As some oils may have possible antiparasitical effect on the skin (the destruction of *T. cruzi* epimastigotes around the bite wound of the Reduviid bug) the potential for adverse reactions need to be investigated.

Eucalyptus oil has been used safely on the skin, however, excessive topical application of the oil can lead to a loss of consciousness, ataxia and muscle weakness which can be reversed by removal of the oil (Darben, Cominos and Lee 1998). In an experiment to treat human facial demodocidosis, eucalyptus oil was used topically in concentrations of 25 – 100% with no side-effects reported suggesting that the topical application of the oil needs to be widespread for its toxic effects to be shown (Morsy, Morsy and Sanand 2002).

TTO has been shown to reduce inflammation induced by histamine in human skin when applied topically, with no adverse reactions (Koh *et al*. 2002) and also reduces the histamine induced oedema in murine ears through the suppression of further histamine release (Brand *et al*. 2002). Conversely, contact dermatitis is not an uncommon occurrence with TTO topical administration with 4 out of 105 human patients experiencing moderate to severe dermatitis in TTO treatment of *Tinea pedis* (Satchell *et al*. 2002). However, there were no reported adverse reaction when TTO was used to treat head lice (Veal 1996) indicating that the majority of people will not experience allergic reactions to this oil and only a small, but significant minority being afflicted (Tisserand & Balacs 1995).

The use of lemon oil topically should be avoided (Tisserand & Balacs 1995). This product contains furocoumarin derivatives that are known to induce photoxicity. Of these, bergapten is the most toxic, followed by oxypeucedanin, which causes photo pigmentation on coloured guinea pig skin without preceding visible erythema

(Naganuma *et al*. 1985). Due to the phototoxic nature of lemon oil, no more than a 2% solution can be applied externally to the skin (Tisserand & Balacs 1995).

1:4 Parasites investigated

Three parasites covering 2 different phyla were used in this study to investigate the antiparasitical actions of 12 plant essential oils (Appendix 1) and 11 soft fruit extracts (Appendix 2). The parasites investigated represent 3 organisms from the World Health Organisations (WHO) neglected diseases initiative which was organised to provide improvements to the health and quality of life for people affected by diseases with expensive and/or lengthy treatment regimens. As these diseases predominantly afflict the very poor, there is no adequate incentive for research and development by pharmaceutical companies (WHO 2003; Savioli, Thompson and Smith 2006). The parasites investigated are shown in Table 1.3 and further described in the text.

Table 1.3 Parasites used in experiments.

PHYLUM	FAMILY	NAME	DISEASE
Sarcomastigophora	Trypanosomatidae	*Trypanosoma cruzi*	American trypanosomiasis
Sarcomastigophora	Hexamitidae	*Giardia duodenalis*	Giardiasis
Apicomplexa	Cryptosporidiidae	*Cryptosporidium parvum*	Cryptosporidiosis

1:5 Giardia duodenalis

The flagellated protozoan parasite *G. duodenalis* was first observed by Antony van Leeuwenhoek in 1681 by investigation of his diarrhoeic stools. *G. duodenalis* (syn. *G. lamblia* and *G. intestinalis*) is a simple binucleated, unicellular organism having a world wide distribution (Ortega & Adam 1997). It is estimated that 280 million humans are infected every year and is the most common intestinal infection in the developed world (Lane & Lloyd 2002; Olson, Ceri and Morck 2000). In developing countries in Asia, Africa and Latin America, an estimated 200 million people have symptomatic giardiasis with 500,000 new cases reported each year (Lane & Lloyd

2002). With industrialised nations, such as the United States of America (USA or US), between 2 – 5% of stool samples submitted for examination can be infected with this organism. In less industrialised countries this Figure can reach 20 – 30% (Marshall *et al*. 1997; Ortega & Adam 1997).

Giardia has 2 major stages in its life cycle, a replicative trophozoite stage and an infective cyst stage. Infection begins with the ingestion of environmentally resistant and infective cysts. After passage through the acidic environment of the stomach and into the proximal small intestine, excystation occurs, releasing 2 trophozoites. The free swimming trophozoites reproduce by binary fission and infect the upper part of the small intestine, particularly the duodenum of their vertebrate hosts, by attachment to the surface of the intestinal wall. Passage of trophozoites to the large colon causes the initiation of encystment in the presence of biliary secretions and slight alkalinity. The environmentally robust cysts are shed in the faeces and are able to infect other hosts upon ingestion.

Trophozoite morphology

The trophozoite of *G. duodenalis* (Figure 1.3 A, B and C) has a pyriform morphology which measures 12 – 15 μm in length, 5 – 9 μm wide, with a rounded anterior end and a pointed posterior end that terminates in 2 flagella (Adam 2001). It is binucleated and bilaterally symmetrical with the 2 oval nuclei located in the anterior end of the parasite (Figure 1.3 A). This provides the parasite, when viewed dorsally under light microscopy, with the appearance of a 'face'. The parasite is dorsoventrally flattened with a convex dorsal surface. Ventrally, the trophozoite surface has a concave, bilobed adhesive disc (ventral disc) which when applied to the host's intestinal cell wall, working in conjunction with the ventral flagella, is responsible for this organisms' ability to further strongly adhere to the host cell surface (Figure 1.3 B & C). This pair of flagella, along with three further pairs of flagella – the anterior, posterior and caudal flagella – arise from kinetosomes located between the anterior portions of the 2 oval nuclei, which are themselves found in the anterior end of the parasite (Figure 1.3 A). The flagellate swims rapidly, swaying from side to side like a falling leaf, a motion also known as 'skipping'.

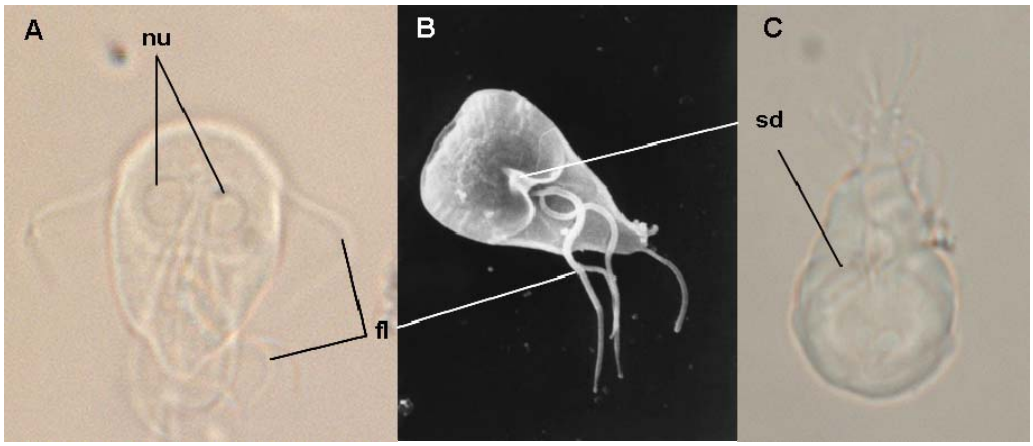


Figure 1.3 Trophozoites of *Giardia duodenalis*.
Live trophozoites were isolated *in vitro* from axenic cultures and viewed under brightfield microscopy (x1000 mag) in ventral view (**C**) and dorsal view (**A**) and ventrally under scanning electron microscopy (**B**). The parasites show classical pyriform morphology and the flagella can be clearly seen in all panels (fl) with the dual nuclei visible in dorsal view (Panel A; nu). The ventral disc is also visible in panel C (sd) and in panel **B** with the ventral flagella easily visible. SEM image courtesy of the Centres for Disease Control and Prevention (CDC) and Dr. J. Carr. Brightfield microscopy images by J-P Anthony (copyright© SPDL).

Cyst morphology

The cyst is produced in the lower bowel and cysts occur in large numbers in the stool. They are oval, measuring approximately 5 by 7 to 10 μm in diameter. Encystation occurs after the trophozoite has undergone nuclear division but before cytokinesis, thus providing the cyst with a single trophozoite containing 4 nuclei (Figure 1.4) and capable of rapidly completing cytoplasmic division upon excystation, producing 2 trophozoites faster than with a normal replication cycle.

A web-like filamentous layer comprises the outer cyst wall with each filament being 7 – 20 nm in diameter (Erlandsen *et al*. 1990; Erlandsen, Bemrick and Pawley 1989) and comprising of four major proteins, 29, 75, 88 and 102 kDa in size (Erlandsen *et al*. 1990) 2 of them being known as cyst wall proteins (CWP) 1 and 2. Underlying this filamentous outer cyst wall is a double membranous layer, the inner membrane layer being separated from the outer membrane by a thin layer of cytoplasm and these membranes separated from the trophozoite plasma membrane by a peritrophic space. It is currently thought that this peritrophic space originates from the

elongation and joining of large cytoplasmic vacuoles from within the trophozoite forming a single clear vacuole that surrounds the cytoplasm of the parasite, pressing against the inner surface of the plasma membrane, ultimately forming the outermost cyst membrane. This process is repeated to form both an inner membrane layer and eventually a new plasma membrane (Chavez-Munguia, Cedillo-Rivera and Martinez-Palomo 2004).

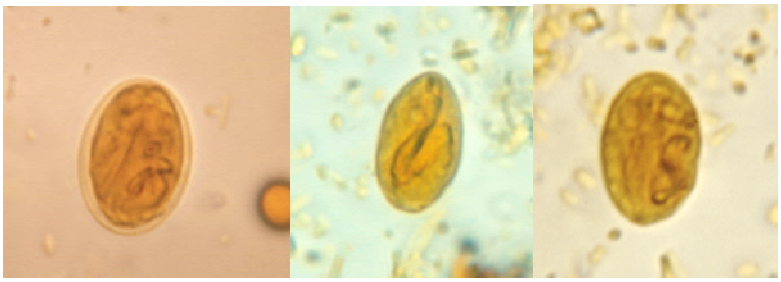


Figure 1.4 Cysts of *G. duodenalis*.
Iodine stained cysts of *Giardia duodenalis* from faecal smears viewed under brightfield microscopy. Cyst formations begin in the jejunum after exposure to biliary secretions and are passed as environmentally robust infective cysts in the faeces. Images courtesy of the CDC.

Protein degradation and trans*port*

Giardia is a diplomonad (of the Order Diplomonadida) and like all members of this order share certain characteristics including 2 karyomastigonts, each having four pairs of flagella, 2 nuclei and lack both mitochondria and a recognizable Golgi apparatus (Adam 2001; Marti *et al*. 2003). A compartment with functional and structural characteristics of the endoplasmic reticulum (ER) has been identified and characterized as an endomembraneous network, which extends throughout the cell body. Other morphologically recognizable membrane compartments are the peripheral vesicles (PVs, approximately 150 – 200 nm in size), which are thought to perform both lysosomal and endosomal activities (Adam 2001).

These PVs are located beneath the plasma membrane of the cell body except at the flagella or where it covers the cytoskeletal structures of the ventral disc. By the uptake of acridine orange, these vesicles were shown to be acidic and by their ability to concentrate exogenous lucifer yellow also demonstrated their endocytic nature (Lanfredi-Rangel *et al*. 1998; Feely, Gardner and Hardin 1991).

Characteristic of most eukaryotes is the ability to degrade and recycle proteins from within the cell or those which are either endocytosed or phagocytosed. Higher eukaryotes have early endosomes responsible for the internalisation of proteins and their subsequent return to the cell membrane or transport to late endosomes. This is followed by their transport to and degradation by lysosomes. Although *Giardia* is a eukaryote, it appears not to have this early / late division of endosomes. This was demonstrated in experiments by Lanfredi-Rangel *et al*. (1998) which showed that when incubated with horseradish peroxidase, the PVs of *Giardia* were labelled rapidly after a few min and that this labelling persisted even after several h.

In the same experiments by Lanfredi-Rangel *et al*. (1998) also suggested a lysosomal function to the PVs because of the localisation of acid phosphatase within most of the vesicles and also in the cisternae of the ER. Glucose-6-phosphatase localisation (a marker of ER) showed the formation of an electron-dense reaction product within the ER but also within a few of the PVs. This suggested that the PVs show continuity with the ER. Earlier experiments (Lindmark 1988; Feely & Dyer 1987) demonstrated the PVs' lysosomal functions through various hydrolase, proteinase and RNAse activities. These experiments suggest that the early/late endosomal and lysosomal system, which act discretely in higher eukaryotes, can be found as a single unit in *Giardia's* peripheral vesicular network and act in concert with the ER.

Although structures consistent with the ER had been described in *Giardia* by EM, it wasn't until experiments by Gupta *et al*. (1994) showed the presence of the BiP gene, a hsp70 (heat shock protein) homologue found in the lumen of the ER in higher eukaryotes, which is responsible for the folding and translocation of proteins across the ER membrane, using molecular techniques and later Soltys, Falah and Gupta (1996) demonstrated that BiP antibodies localised to ER-like structures that the presence of an ER was confirmed.

The presence of three protein-disulphide isomerase genes (gPDI-1, 2 and 3) and their expression provided further evidence of an ER within *Giardia* as these enzymes are known to be within the lumen of the ER (Knodler *et al*. 1999). A major function of

the ER is the formation and folding of disulphide bonds for membrane and secreted proteins. When correct disulphide bond formation is inhibited in the ER by dithiothreitol (DTT; a reductant which interferes with the redox environment of the ER), protein modification and transport can be prevented. In higher eukaryotes, this causes a reduction in protein glycosylation. *Giardia*, however, has very few secreted glycosylated proteins (Adam 1991). Nevertheless, DTT can inhibit the formation of disulphide bonds in cyst wall proteins, inhibiting the differentiation of *Giardia* trophozoites into cysts suggesting a role for gPDIs in this process (Knodler *et al*. 1999).

Encystation

The encystation process generally begins in the jejunum after exposure to biliary secretions. By mimicking these conditions, it has been possible to perform encystation of *G. duodenalis in vitro* (Gillin *et al*. 1987; Gillin, Reiner and Boucher 1988; Schupp *et al*. 1988). A slightly alkaline pH (7.8) was required to generate cysts resistant to hypotonic conditions as found in faeces (Gillin *et al*. 1987). Other important factors were the presence of bile salts and saturated fatty acids (Gillin *et al*. 1987; Gillin, Reiner and Boucher 1988).

Once encystation is initiated, the process can be completed within 26 h and involves 2 phases, an early phase (completed within 10 h) and a late phase (completed within 16 h). During the early phase, intracellular synthesis and transport of the cyst wall proteins occur. At this time, a Golgi-like stack of membranes arise and are associated with the development of cyst wall antigens and their transport via large (up to 1 μm), osmophillic, secretory vesicles known as encystation-specific vesicles (ESVs) (Reiner, McCaffery and Gillin 1990). There is increasing evidence that ESVs may be the cisternae of an unique Golgi equivalent in *Giardia* (Adam 2001; Hehl & Marti 2004; Luján *et al*. 1995; Marti *et al*. 2003). As these ESVs appear during encystation it is possible that they are newly generated at this time and are not constitutively expressed. Experiments by Marti *et al*. (2003) however, found a lack of regulated expression of Golgi apparatus associated proteins suggesting that, at the molecular level at least, the secretory system of *Giardia* is not stage-regulated. Further to this,

antibodies generated against proteins of the endomembrane system showed that cyst wall proteins were exported through the re-organisation membrane compartments that were biochemically similar to ESVs (Marti *et al*. 2003). These results demonstrate ESVs are part of a Golgi cisternae-like system which is constitutively expressed rather than being generated *de novo*.

Excystation

Within the mammalian host, excystation occurs rapidly after passage through the acidic environment of the stomach and after entry into and exposure to elements of the proximal small intestine (Adam 2001). Excystation was first induced *in vitro* using acidic solutions (optimum pH 1.3 – 2.7; Bingham & Meyer 1979) which suggest an importance of passage through the acidic environment of the stomach for excystation. This may not always be the case, with *G. muris* excystation having occurred *in vitro* at pH 7.5 *in vitro* (Feely, Gardner and Hardin 1991) and may explain cases of giardiasis in patients with reduced gastric acidity. Subsequently a pH of 4 has been assessed as the optimum pH and that the use of pancreatic proteases such as trypsin or chymotrypsin being required for *in vitro* excystation and suggest the role for pancreatic secretions for excystation *in vivo* (Boucher & Gillin 1990).

Life cycle

Humans are indicated as reservoirs of this protozoan, although other mammals including dogs, cats, farm animals and beavers have been shown to harbour *Giardia* (Ortega & Adam 1997; McGlade *et al*. 2003; Hunter & Thompson 2005). Transmission in humans may occur via direct faecal-oral contact or from faecally contaminated water and food (Smith *et al*. 2007). The zoonotic transmission of *Giardia* is still unclear but has been described in northeast India from dogs (Traub *et al*. 2004) and direct contact with farm animals and pets have also been associated with giardia infection (Warburton, Jones and Bruce 1994; Hunter & Thompson 2005).

The infection cycle begins with the consumption of infective cysts, usually from the faecal-oral route by contaminated food or water (Figure 1.5). After the safe passage

of the cysts through the stomach excystation occurs, releasing 2 trophozoites into the lumen of the proximal small intestine. The trophozoites divide by binary fission, beginning with the nuclear division, followed by the locomotor apparatus, the ventral disc and finally the cytoplasm. In this way, large numbers of trophozoites are rapidly generated within the lumen of the small intestine. Whilst trophozoites may be found in the profuse watery stools of symptomatic infection (reviewed by Dawson 2005), only the cysts are capable of surviving out with the host. Cyst formation occurs with the passage of trophozoites, in conjunction with intestinal contents, to the colon. Exposure of trophozoites to the slightly alkaline environment (pH 7.8) of the colon and to biliary secretions such as bile salts and saturated fatty acid causes the induction of encystment. These cysts are shed in the faeces and are resilient to the environment, remaining viable in lake water for up to 56 days, in tap water for up to 14 days (de Regnier *et al*. 1989) and 2 – 3 months at temperatures below 10°C (WHO 2004).

Clinical Manifestations in Man

Giardiasis has an incubation period of anywhere between 5 and 25 days with approximately 60% of infected individuals being asymptomatic (Ortega & Adam 1997). In the remaining 40% of individuals, symptoms usually commence 1 – 2 weeks after infection and will normally last 2 – 6 weeks with the diarrhoea improving after approximately 1 week. In this acute phase, symptoms include: nausea, anorexia, malaise, possible discomfort in the upper intestine and possibly a low grade fever and chills. Following from these symptoms are the sudden onset of explosive, watery, foul smelling diarrhoea and associated with abdominal distension and an increase in foul flatulence. Cramping of the upper to mid-epigastria may occur accompanied with foul belching. This acute stage may last only for a few days in immunocompetent hosts and the infection will commonly resolve spontaneously. However, the acute stage has been known to last months leading to malabsorption, steatorrhoea (fatty stools), debility and weight loss. Some patients may become asymptomatic cyst passers for a period and will display no other clinical manifestations.

Following on from the acute stage is the development of a chronic or subacute stage of infection. Typically the patient exhibits recurrent and brief episodes of loose stools (which may be frothy, yellowish and float on the toilet water) accompanied by foul flatus and increased abdominal distension. Not uncommon is a condition called the 'purple burps' where belching is accompanied with a rotten egg taste. Anorexia and nausea are common as well as fatigue, headaches and myalgias possibly occurring. These symptoms are alleviated following effective drug treatment.

Treatment

In the majority of cases, a short treatment regimen of tinidazole (Fasigyn®) (2 g once in adults) or metronidazole (Flagyl®) (250 mg three times daily 7 days for adults) are 80% to 95% effective, respectively (Petri 2005). However, side effects are common with these drugs and are described as nausea, headaches, diarrhoea, abdominal cramps, a strong metallic taste, dizziness, dark discoloured urine and a disulphiram-like reaction when taken with alcohol (nausea and vomiting) (Harris, Plummer and Lloyd 2001; Ortega & Adam 1997).

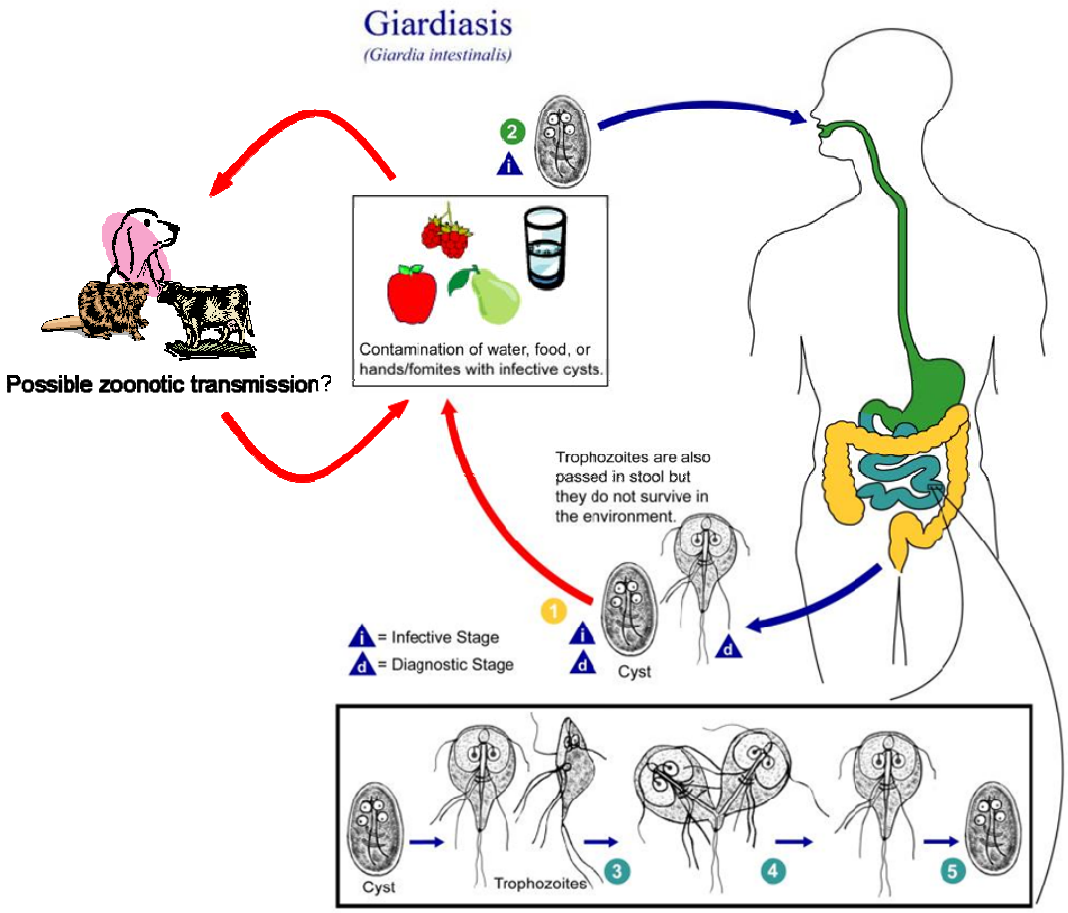


Figure 1.5 Life-cycle of *G. duodenalis*.
Cysts are resistant forms and are responsible for transmission of giardiasis. Both cysts and trophozoites can be found in faeces (diagnostic stages)1. The cysts are hardy and can survive several months in cold water. Infection occurs following the ingestion of cysts in contaminated water, food, or by the faecal-oral route (hands or fomites)2. In the small intestine, excystation releases trophozoites (each cyst produces 2 trophozoites)3. Trophozoites multiply by longitudinal binary fission remaining in the lumen of the proximal small bowel where they can be free or attached to the mucosa by a ventral sucking disk4. Encystation occurs as the parasites transit toward the colon. The cyst is the stage found most commonly in non-diarrhoeal faeces5. Because the cysts are infectious when passed in the stool or shortly afterward, person-to-person transmission is possible. While animals such as cattle, beavers and dogs are infected with *Giardia*, their importance as a reservoir and zoonotic source of transmission is unclear. Source: Figure adapted from the Public Health Image Library – National Institutes of Health and the CDC.

1:6 Cryptosporidium parvum

Cryptosporidium parvum is an enteric protozoan parasite first identified in 1976 (Nime *et al*. 1976; Meisel *et al*. 1976) as causing gastrointestinal disease in humans, characterised by self limiting diarrhoea in immunocompetent hosts and chronic, severe diarrhoea and potentially fatal consequences in immunocompromised hosts (Hunter & Nichols 2002; Farthing 2000). Since the discovery of human cryptosporidiosis many human outbreaks have occurred in immunocompetent humans. In the period 1983 – 2003 there has been an estimated 504,019 individuals infected with *Cryptosporidium* oocysts in 165 documented waterborne outbreaks, worldwide (Karanis, Kourenti and Smith 2007; Smith & Rose 1998). The largest waterborne outbreak to date has been the 1993 outbreak in Milwaukee, Wis. USA where over 400,000 people were estimated to be infected (MacKenzie *et al*. 1994a and 1994b), although due to the reporting methods employed to ascertain infection in that particular outbreak (telephone enquiries and self-reporting of diarrhoeal symptoms) this figure could have been over estimated (Hunter & Syed 2001).

Of the 19 known valid species and 40 genotypes of *Cryptosporidium*, 8 species (*C. hominis*, *C. parvum*, *C. meleagridis*, *C. felis*, *C. canis*, *C. suis*, *C. andersoni* and *C. muris*) and 5 genotypes of *Cryptosporidium* (chipmunk, cervine [2 of 3 genotypes], monkey, and skunk) can infect humans. Of the 8 species, *C. hominis* and *C. parvum* are responsible for the majority of human disease (Table 1.4; Smith 2008; Feltus *et al*. 2006; Leoni *et al*. 2006; Nichols, Campbell and Smith 2006; Caccio *et al*. 2005; Ryan *et al*. 2004; Xiao *et al*. 2004). *C. parvum* has been the species most studied with respect to infectivity *in vivo* and viability *in vitro*. *C. parvum*, which can be cultured *in vitro*, has also been the species used mostly for drug discovery investigations and as such was used in experiments for this thesis and shall be discussed in this introduction. The parasite has a complex life cycle that involves both a sexual and asexual reproductive cycle which can be completed within a single host beginning with the ingestion of an infective oocyst and the eventual passing of further oocysts in the faeces of the infected host. The oocyst is environmentally robust and chlorine insensitive, which coupled with its small size make it possible for it to survive water treatment.

Cryptosporidium is an apicomplexan parasite of the Order Eucoccidiorida and forms oocysts containing 4 sporozoites which are not contained within a sporocyst. The sporozoites when excysted, infect the epithelia of the intestinal tract (enterocytes) of various mammals (Smith, Nichols and Grimason 2005). *In vitro* and *in vivo* investigations into *C. parvum* excystation have highlighted some of the host- and parasite-derived triggers required to initiate infection, although the process of excystation is still poorly understood.

Morphology of the oocyst

When viewed under Nomarski differential interference contrast (DIC) microscopy oocysts of *C. parvum* appear spherical or slightly ovoid, smooth, thick walled and refractile measuring 4.5 – 5.5 µm in diameter. Each oocyst contains 4 elongated and curved naked sporozoites, although all 4 sporozoites may not be visible at the same time (Figure 1.6).

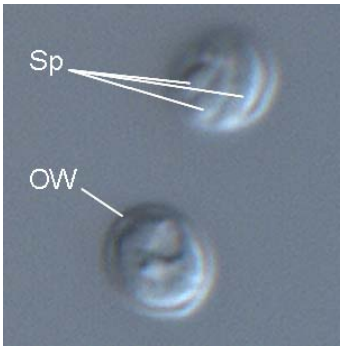


Figure 1.6 Oocysts of *C. parvum*.
Oocysts of *C. parvum* viewed under Nomarski DIC (x1250 total magnification) showing three out of four sporozoites (Sp) in a typical 'finger' arrangement and the thick, protective oocyst wall (OW). Nomarski DIC image taken by J-P Anthony (copyright© SPDL).

The sizes of *Cryptosporidium* oocysts are varied and one cannot always rely on oocyst size alone as a means of identification of species, as can be seen in Table 1.4. A more reliable form of identification between species is through genetic characterisation as determined by polymerase chain reaction, restriction fragment length polymorphism and DNA sequencing analyses at 2 18S rRNA loci (Nichols, Campbell and Smith 2003; Nichols, Campbell and Smith 2006; Xiao *et al*. 2000a;

Xiao *et al*. 1999), the *Cryptosporidium* oocyst wall protein (COWP) locus (Homan *et al*. 1999; Xiao *et al*. 2000b), thrombospondin-related adhesive protein 1 (TRAP-C1; (Spano *et al*. 1998)) and β-tubulin (Caccio *et al*. 1999; Rochelle *et al*. 1999; Sulaiman, Xiao and Lal 1999; Widmer *et al*. 1998).

Table 1.4 Characteristics of recognized *Cryptosporidium* species.

Species of *Cryptosporidium*	Major Host	Oocyst Dimensions (µm)	Site of Infection	Reference
C. andersoni	Cattle	5.5 x 7.4	Abomasum	(Lindsay *et al*. 2000)
C. baileyi	Poultry	4.6 x 6.2	Bursa of Fabricus, cloaca and trachea	(Current, Upton and Haynes 1986)
C. bovis	Cattle	4.7-5.3 x 4.2-4.8	Small intestine	(Barker & Carbonell 1974)
C. canis	Dogs	4.95 x 4.71	Small intestine	(Fayer *et al*. 2001)
C. fayeri	Red kangaroo (*Macropus rufus*)	4.5-5.1 × 3.8-5.0	Intestine	(Ryan, Power and Xiao 2008)
C. felis	Cats	4.5 x 5.0	Small intestine	(Asahi *et al*. 1991; Iseki 1979)
C. galli	Finches, Chickens	8.25 x 6.3	Proventriculus	(Ryan *et al*. 2003; Pavlasek 2001 & 1999)
C. hominis	Humans	4.5 x 5.5	Small intestine	(Morgan-Ryan *et al*. 2002)
C. macropodum	Grey kangaroo (*Macropus giganteus*)	Not known	Not known	(Power & Ryan 2008)
C. meleagridis	Turkeys	4.5-4.0 x 4.6-5.2	Small intestine	(Slavin 1955)
C. molnari	Fish	4.7 x 4.5	Small intestine and stomach	(Fayer 2008; Alvarez-Pellitero & Sitjà-Bobadilla 2002)
C. muris	Rodents	5.6 x 7.4	Stomach	(Tyzzer 1907)
C. parvum	Man and other mammals	4.5 x 5.5	Small intestine	(Tyzzer 1912)
C. ryanae (previously *Cryptosporidium* deer-like genotype)	Cattle (*Bos taurus*)	2.94-4.41 x 2.94-3.68	Not known	(Fayer, Santin and Trout 2008; Santin *et al*. 2004)
C. scophthalmi	Fish (Turbot)	3.7-5.0 x 3.0-4.7	Intestine, very seldom in the stomach	(Alvarez-Pellitero *et al*. 2004)
C. serpentis	Snakes, Reptiles	5.6-6.6 x 4.8-5.6	Stomach, gastric glands	(Levine 1980)
C. suis	Pigs	4.9-4.4 x 4.0-4.3	Small and large intestine	(Ryan *et al*. 2004)

Table 1.4 – Continued

Species of *Cryptosporidium*	Major Host	Oocyst Dimensions (µm)	Site of Infection	Reference
C. varanii (previously *C. saurophilum*	Lizards	4.2-5.2 x 4.4-5.6	Stomach and small intestine.	(Pavlasek & Ryan 2008; Koudela & Modry 1998)
C. wrairi	Guinea pigs	4.9-5.0 x 4.8-5.6	Small intestine	(Vetterling & Jervis 1971; Vetterling, Takeuchi and Madden 1971)

At least 8 species (*C. hominis*, *C. parvum*, *C. meleagridis*, *C. felis*, *C. canis*, *C. suis*, *C. andersoni* and *C. muris*) and 5 genotypes *Cryptosporidium* (chipmunk, cervine [2 of 3 genotypes], monkey, and skunk) can infect humans, but it is *C. parvum* and *C. hominis* which are responsible for the majority of human instances of cryptosporidiosis (Feltus *et al*. 2006; Leoni *et al*. 2006; Nichols, Campbell and Smith 2006; Caccio *et al*. 2005; Ryan *et al*. 2004; Xiao *et al*. 2004). Table adapted from Smith (2008) and Smith *et al*. (2007).

Oocyst wall structure

The oocysts of *Cryptosporidium* are extremely resistant to environmental pressures (Nichols, Paton and Smith 2004; Robertson, Campbell and Smith 1992) and disinfectants (Angus *et al*. 1982; Blewett 1989; Campbell *et al*. 1982; Korich *et al*. 1990; Li *et al*. 2004; Pavlasek 1984; Peeters *et al*. 1989). The oocyst wall is the interface between the host, the oocyst and the environment and is composed of 3 main layers: outer, central and inner layers and measured to be 40 – 50 nm thick (Harris & Petry 1999; Nanduri *et al*. 1999). Coating the oocyst wall is a carbohydrate-rich glycocalyx, 20 – 30 nm thick and is antigenic (Nanduri *et al*. 1999).

The outer layer of the oocyst wall is composed of acidic glycoproteins and lipids between 5 and 10 nm thick which is variably electron dense (Harris & Petry 1999; Robertson, Campbell and Smith 1993a). A 5 nm lipid-rich central layer separates the outer and inner layers of the oocyst wall. Finally there is an inner layer which is 30 nm thick and composed of 2 further layers: a 10 nm electron dense outer-inner layer and a 20 nm less electron dense inner-inner layer. With the 10 nm thick outer-inner layer a typical fibrillar array can be seen in negatively stained sections as disordered filamentous material (Smith & Ronald 2002). A protease sensitive, 20 nm thick,

cross-linked filamentous glycoprotein layer comprises the inner-inner layer (Bonnin, Dubremetz and Camerlynck 1991) and along with the central lipid layer, provides much of the structural rigidity and elasticity of the oocyst wall (Harris & Petry 1999). The inner layer of *C. parvum* oocysts also contains a unique feature of a linear suture which spans one third to one half of the circumference of the oocyst wall which allows the exit of sporozoites during the excystation process.

Oocyst sources for experimentation

Oocysts are most commonly purchased commercially for use in experimental research. These will come from various infected animals and in the case of *C. parvum*, cattle. *In vitro* studies have been successful in culturing *C. parvum* in enterocytes and have been invaluable in the investigation of *Cryptosporidium's* life cycle (Current & Haynes 1984; Current & Reese 1986). Recently, there have been some controversial advances in the field of *in vitro* culture where it has been claimed that the life cycle of *C. parvum* has been successfully completed without the requirement of the enterocyte (Hijjawi *et al*. 2004, 2002 and 2001). Replication of this experiment by others, however, has proven difficult (Girouard *et al*. 2006).

Life cycle

Asexual reproduction

Infection begins through the ingestion of infective, sporulated oocysts. In the case of *C. parvum* a remarkably low infectious dose can initiate infection (dose required to cause infection in 50% of test subjects; ID_{50} = 9 – 1042 oocysts; (Okhuysen *et al*. 1999)). Four naked, motile sporozoites are released through the suture of the oocyst in response to temperature (37^{o}C), pH fluctuations, bile salts, reducing agents, proteases and time (Fayer & Leek 1984; Kato *et al*. 2001; Robertson, Campbell and Smith 1993b; Smith, Nichols and Grimason 2005). Exposure to acid (pH ~2) followed by incubation in bile salts, reducing agents and proteases, mimicking transit through the acidic stomach to the alkaline small intestine, enhances excystation *in vitro*, but the fact that oocysts can excyst *in vivo* and cause disease in extraintestinal locations indicates that some of these host-derived triggers are not essential (Smith, Nichols and Grimason 2005).

Upon excystation, sporozoites attach to the luminal surface of enterocytes and rapidly invade these cells with the complete process of location, attachment, invasion and internalisation occurring within 25 min of excystation with *C. parvum* sporozoites (Langer & Riggs 1999).

The sporozoite, upon locating the surface of the enterocyte, will orientate itself so that its anterior pole is towards the surface, with ligand-receptor interactions mediating attachment (Smith, Nichols and Grimason 2005). As the sporozoite penetrates the cell, the apical membrane of the enterocyte extends and protrudes over the apical end of the parasite. Complete engulfment of the parasite follows, with the sporozoite contained within a parasitophorous vacuole comprising of sporozoite plasma membrane and enterocyte apical membrane (Chen *et al*. 1998). This vacuole whilst intracellular, is separated from the cell cytoplasm by an electron-dense structure of host cellular elements such as actin (Chen *et al*. 2002; Clark 1999; Elliot *et al*. 2001; Tzipori & Griffiths 1998).

The sporozoites differentiate into round trophozoites which, after nuclear division, results in the development of multinucleated schizonts sometimes known as meronts (schizogony). There are 2 types of schizonts; type I and type II. The type I schizont is involved in the asexual reproduction of *C. parvum* and is distinguishable from type II schizonts by containing between 6 and 8 nuclei which develop into 6 – 8 type I merozoites which are small in size (1 x 1.2 μm) and generally round to oval in shape (Current & Reese 1986; Hijjawi *et al*. 2004, 2002 and 2001). A type II schizont will contain 4 merozoites which can vary in morphology between being spindle shaped with pointed ends (3.5 x 2 μm) and round or oval (1.6 x 1.5 μm) (Current & Reese 1986; Hijjawi *et al*. 2004, 2002 and 2001). Release of the actively motile merozoites from type I schizonts can infect neighbouring cells and undergo a replicative cycle similar to that already described for trophozoites, thus providing further type I merozoite progeny and a means of re-infection, or they can develop into type II schizonts.

Sexual reproduction

A mature type II schizont contains 4 type II merozoites which can initiate the sexual multiplication cycle (gametogony). Each individual merozoite produces either microgamonts or macrogamonts. The microgamont is large (5.6 x 5 μm) and spherical in shape and nuclear division in the microgamont produces numerous microgametes (Current & Reese 1986; Hijjawi *et al*. 2004, 2002 and 2001). The motile microgametes (oval shaped with a large nucleus filling most of the cytoplasm; 2.2 x 1.6 μm) are released from the parasitophorous vacuole and each can fertilise a macrogamont (5 x 4 μm, containing a peripheral nucleus) (Current & Reese 1986; Hijjawi *et al*. 2004, 2002 and 2001). The product of fertilisation (zygote) develops into an oocyst. Sporozoite generation occurs in the zygote in a process of sporogony and most zygotes (approximately 80%) will become fully sporulated oocysts (containing 4 sporozoites each) to be released in the intestinal lumen and from there pass into the faeces (Current & Garcia 1991; Current& Reese 1986). These are infectious to other susceptible hosts when excreted. However, autoinfection of the host can also occur with approximately 20% of the zygotes failing to form a thick oocyst wall (Current & Garcia 1991; Current & Reese 1986). The sporozoites in these sporulated oocysts are contained within a "single unit membrane" which, upon release from the host cell, ruptures releasing the sporozoites into the intestinal lumen (Current & Garcia 1991; Current & Reese 1986). These released sporozoites are able to reinfect the host enterocytes and begin the process of schizogony, gametogony and sporogony again (Current & Garcia 1991; Current 1988; Current & Reese 1986; Fayer 2007; Smith, Nichols and Grimason 2005).

Large numbers of oocysts can be generated in the host and approximately 10^{10} oocysts can be excreted by an infected symptomatic host (Karanis, Kourenti and Smith 2007). The complete life-cycle can be seen in Figure 1.7.

Clinical Manifestations in Humans

The most frequently recognized clinical picture of cryptosporidiosis involves an enteric infection with chronic, watery and often debilitating diarrhoea in more than 90% of immunocompetent patients (Hunter & Nichols 2002; Farthing 2000;

MacKenzie *et al*. 1994b). However, asymptomatic infection also occurs (Farthing 2000). A non-specific low-grade fever, malaise, anorexia, abdominal discomfort and nausea may accompany the diarrhoea, but no clinical or characteristic laboratory feature other than identification of the organism or oocyst in a clinical specimen can distinguish the cause of the illness. In an immunocompetent host, cryptosporidiosis is usually acute and self-limiting with the symptoms lasting 1 - 2 weeks. These symptoms, if chronic, can lead to weight loss, dehydration and malnutrition (Juranek 1995; MacKenzie *et al*. 1994b). The severity of the illness varies but appears to be directly related to the patient's immunocompromised state. Immunocompetent persons usually experience a short-term diarrhoeal illness when symptomatic, whereas a prolonged cholera-like illness may be seen in severely immunosuppressed persons, such as those with autoimmune deficiency syndrome (AIDS). Improvements to human immunodeficiency virus (HIV) treatment such as the highly active antiretroviral therapy (HAART), which suppresses HIV replication and increases circulating CD4 T lymphocyte cells (Ho *et al*. 1995; Wei *et al*. 1995), has led to a reduction of cryptosporidiosis among HIV-positive patients (reviewed by Smith & Corcoran 2004; Chen *et* al. 2002; Schmidt *et al*. 2001). Whilst *C. parvum* infection is normally confined to the gastrointestinal tract, cryptosporidiosis has been identified in the biliary tract (causing thickening of the gallbladder wall), the respiratory system, middle ear, pancreas and stomach, principally in AIDS patients (Casemore, Gardner and O'Mahony 2007; Clark 1999; Farthing 2000).

Treatment

Despite the fact that more than 200 drugs have been tested for their anti-cryptosporidial effects (Mead 2002) *in vitro* (e.g. growth inhibition in CaCo2 and HCT-8 cells; (Theodos *et al*. 1998)) and *in vivo* (infectivity studies in mice and pigs; (Tzipori 1998)) effective, specific drug treatment for cryptosporidiosis remains a goal. This is not to say, however, there is no effective drug therapy for *Cryptosporidium* infection. Nitazoxanide has been demonstrated to have efficacy for cryptosporidiosis with immunocompetent patients, inhibits *C. parvum* growth *in vitro* and has been licenced for paediatric use in the USA (Rossignol 2006; Bailey & Erramousepe 2004; Smith & Corcoran 2004; Amadi *et al*. 2002; Theodos *et al*.

1998). This drug has demonstrated clinical (resolution of diarrhoea) and parasitological (absence of *Cryptosporidium* in stools) response rates of 80% and 70%, respectively with immunocompetent patients (Bailey & Erramousepe 2004). Lower response rates were seen with immunocompromised patients. In June 2004, nitazoxanide was also licenced by the US Food and Drug Administration (FDA) for use in all persons ≥ 1 year of age.

As chronic cryptosporidiosis is more likely to occur in those patients with lower CD4 T cell counts (Elliot *et al*. 1997; Hashmey *et al*. 1997; Navin *et al*. 1999; Pozio *et al*. 1997) and that HAART allows the recovery of CD4 T cells (Ho *et al*. 1995; Wei *et al*. 1995) it has been shown that this therapy can reduce the incidence of cryptosporidiosis in HIV-postive patients (reviewed by Smith & Corcoran 2004). Investigations by Hommer *et al*. (2003) have demonstrated that the protease inhibitors used in HAART reduce enterocyte invasion by *Cryptosporidium* sporozoites and also reduce the development of sporozoites *in vitro*. The combination of nitazoxanide and HAART has been suggested as a possible treatment for cryptosporidiosis in immunocompromised patients (Smith & Corcoran 2004). As cryptosporidiosis is, in most cases, a self-limiting illness in immunocompetent individuals, general, supportive care is the only treatment that is required. Oral or intravenous rehydration and replacement of electrolytes may be necessary for those with particularly voluminous, watery diarrhoea (Flanigan & Soave 1993).

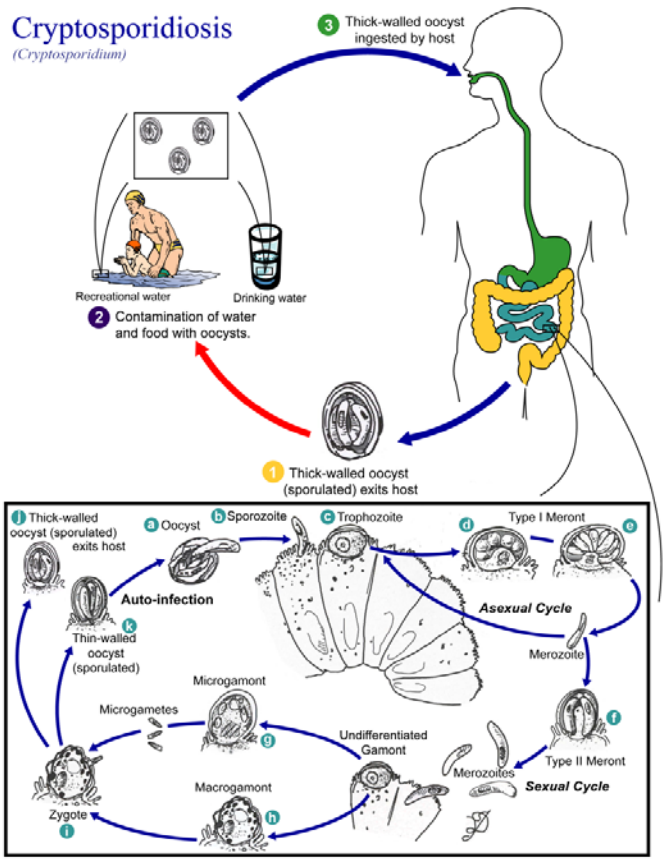


Figure 1.7 Life-cycle of *C. parvum*.
Sporulated oocysts, containing 4 sporozoites, are excreted by the infected host through faeces and possibly other routes such as respiratory secretions(1). Transmission of *Cryptosporidium parvum* occurs mainly through contact with contaminated water (e.g., drinking or recreational water). Occasionally food sources may serve as vehicles for transmission. Zoonotic and anthroponotic transmission of *C. parvum* occur through exposure to infected animals or exposure to water contaminated by faeces of infected animals(2). Following ingestion (and possibly inhalation) by a suitable host(3), excystation (a) occurs. The sporozoites are released and parasitize epithelial cells ((b),(c)) of the gastrointestinal tract or other tissues such as the respiratory tract. In these cells, the parasites undergo asexual multiplication (schizogony or merogony) ((d), (e), (f)) and then sexual multiplication (gametogony) producing microgamonts (male) (g) and macrogamonts (female) (h). Upon fertilization of the macrogamonts by the microgametes ((i)), oocysts ((j),(k)) develop that sporulate in the infected host. Two different types of oocysts are produced, the thick-walled, which is commonly excreted from the host(j) and the thin-walled oocyst(k), which is primarily involved in autoinfection. Oocysts are infective upon excretion, thus permitting direct and immediate faecal-oral transmission. **Source:** Public Health Image Library – CDC and Alexander J. da Silva/Melanie Moser 2002. *Cryptosporidium* stages were reproduced from Juranek DD. Cryptosporidiosis. In: Strickland, G.T., editor. 2000. Hunter's Tropical Medicine and Emerging Infectious Diseases, 8th ed. Philadelphia: WB Saunders. Originally adapted from the life cycle that appears in Current, W.L. and Garcia. L.S. 1991. Cryptosporidiosis. *Clinical and Microbiological Reviews*. vol. 4, pp. 325-58.

1:7 Trypanosoma cruzi

This flagellated protozoan parasite, described by Carlos Chagas in 1909 (Chagas 1909) from dissected cone-nosed bugs (Reduviid bugs), was not associated with disease (American trypanosomiasis) until several years later. The disease caused by this parasite is also known by the name of its discoverer and Chagas' disease is of major medical importance throughout Latin America with 16 – 18 million people infected, up to 120 million at risk of infection (25% of the population of Latin America) 25 – 30% of those infected chronically suffering irreversible damage to the heart and digestive tract leading to approximately 50,000 fatalities every year (WHO 2002). Infection by this parasite presents acute and chronic symptomology. During the acute phase of infection a patent parasitaemia lasting 2 – 3 months occurs which is usually asymptomatic, but can present with manifestations that include fever, anorexia, lymphadenopathy, mild hepatosplenomegaly and myocarditis. Due to the infection often being asymptomatic at his stage, the disease is often not diagnosed.

After this acute phase there is a chronic phase of infection which has an indeterminate period of infection lasting from several years to persisting indefinitely with varied symptoms ranging from cardiomyopathy, peripheral nervous system damage or dysfunction of the digestive tract. If untreated, the disease can be fatal, usually due to heart attacks brought on by cardiomyopathy. At present there is no known cure for chronic Chagas' disease, only for the alleviation of the clinical manifestations. Chronically infected human hosts living in endemic regions form an important parasite reservoir with the life cycle alternating between vertebrate and insect hosts. The replicative epimastigote and infective metacyclic trypomastigotes are found in the blood-sucking arthropod vectors (the Reduviid bugs) with the intracellular and replicative amastigotes and bloodstream trypomastigotes in the mammalian host.

Trypanosoma cruzi is a flagellate of the order Kinetoplastida, Family Trypanosomatidae, characterized by the presence of one flagellum and a single mitochondrion in which is situated the kinetoplast, a specialized DNA-containing organelle. During its life-cycle, the parasite developes into different forms which are

identified by the relative position of the kinetoplast in relation to the cell nucleus and emergence of the flagellum from the cell body.

Morphology in the Mammalian Host

Trypomastigote

The trypomastigote stages of the parasite can be found in the circulating blood and 2 morphological types of bloodstream trypomastigotes have been observed. One is slender, with an elongated nucleus, a subterminal kinetoplast and a short free flagellum. The other is broad, with an oval nucleus, an almost terminal kinetoplast and with a long free flagellum. It has been postulated that the slender forms are responsible for infection in the vertebrate host, with the broad forms infecting the invertebrate.

In most cases a slender form is found, being 16 – 20 μm long with a pointed posterior end (Figure 1.8 A). It contains a large mitochondrion which extends throughout the length of the cell with a kinetoplast within it that is located sub terminally at the posterior end of the parasite which can often cause the cell body to 'bulge' around it and positioned to the basis of the flagellum. The kinetoplast is rod-like in shape and contains ~30% of the parasites total DNA and is known as kinetoplast DNA (k-DNA). The nucleus is elongated, located anteriorly to the kinetoplast and is almost central within the cell body. A single flagellum is attached to the basal body and emerges from a specialised invagination, the flagellar pocket. This pocket surrounds the kinetosome from which the flagellum arises and is found closely associated with the kinetoplast. The flagellum runs along the surface of the cell body and continues past as a 'free whip' anterior to the cell and is able to propel the organism in an undulating fashion. An undulating membrane is formed with the association of the flagellar membrane which is closely apposed to the cell body surface and the beating action of the flagellum. As the flagellum beats the close association of the flagellar membrane to the cell surface pulls this area of the pellicle into a fold and this fold in conjunction with the flagella constitute the undulating membrane. The flagellum is important not only for movement but also for the attachment of the parasite to the surface of mammalian host cells and to the intestinal

perimicrovillar membranes in the invertebrate host. Attachment of the trypomastigote leads to the invasion of the cell by the parasite and the eventual differentiation of the trypomastigote into the replicative intracellular amastigote form. It is the ability of this parasite to invade a wide range of nucleated cells in the mammalian host, allowing it to infiltrate and replicate in privileged sites that makes treatment of Chagas' disease difficult.

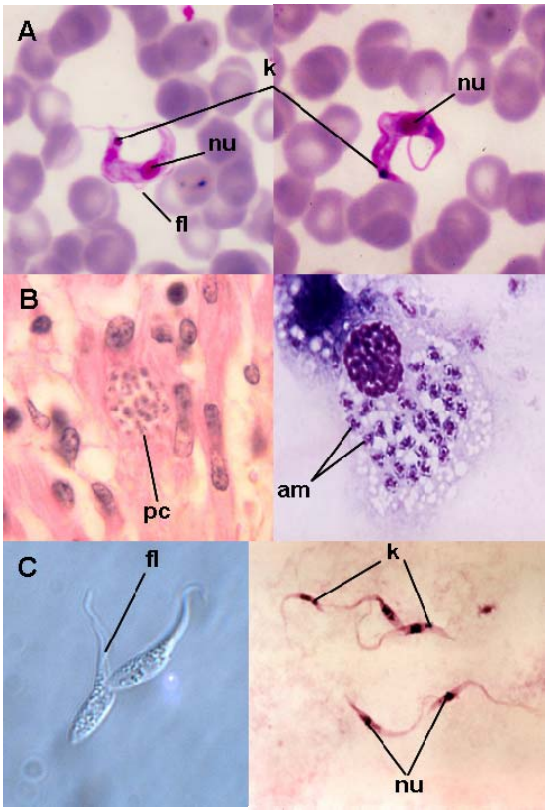


Figure 1.8 Life-cycle stages of *T. cruzi*.
Various life cycle stages of *T. cruzi* showing geimsa stained trypomastigotes in a blood smear (**A**) showing the centrally located nucleus (nu) anterior to the kinetoplast (k) which is located at the posterior end and the single long flagellum is also visible (fl). Amastigotes in a pseudocyst (pc) are visible in a geimsa stained section of monkey heart (**B – left hand side**) with a single HeLa cell filled with amastigotes (am) (**B –right hand side**). The insect forms are shown in panel **C** and can be cultured axenically *in vitro*. The short flagellum is visible on the left hand image (Nomarski DIC, x1250 mag.) and in Geimsa stained epimastigotes the basket shaped kinetoplast (k) is located anteriorally to the round and central nucleus (nu). Images are courtesy of the CDC (Geimsa stained epimastigotes) and Dr. Mae Melvin (trypomastigotes); Dr. L.L. Moore Jnr. (amastigotes in monkey cardiac muscle); Dr. A.J. Sulzer (amastigotes in HeLa cell); J-P Anthony (copyright© SPDL; epimastigotes under Nomarski DIC).

Amastigote

These replicative stages of the parasite develop rapidly after invasion of the trypomastigote. Invasion occurs initially through the formation of a membrane bound vacuole which will subsequently be disrupted releasing the parasite into the cytoplasm. The vacuole is acidified by the fusion of lysosomes and the parasite secretes a porin-like molecule, Tc-Tox, which facilitates parasite exit from the vacuole into the cytoplasm of the host cell (Andrews 1993). It is after this transition that the parasite differentiates into the amastigote stage with the proliferation of amastigotes causing the formation of a pseudocyst (Andrews 1993). The parasite shortens in length and becomes spheroid between 3 – 5 μm in diameter (De Souza 2002; Tyler & Engman 2001). At this time the flagellum is internalised, projecting only slightly beyond the flagellar pocket (Figure 1.8 B). The kinetoplast also changes morphology from a rounded, basket-like shape with a more open and less organised k-DNA seen in the trypomastigote, to a rod-like structure with tightly packed k-DNA, which remains basally to the flagellum. A rounded nucleus can also be observed which, as in trypomastigotes, is found in a central position in the cell body, anterior to the kinetoplast. When ingested along with trypomastigotes in a Reduviid bug blood meal, they can transform in the insect mid gut to form epimastigotes. The cell body begins to swell and the flagellum lengthens and begins to beat. At this point they are known as sphaeromastigotes and as the cell density increases, the cell body lengthens further as does the flagellum to become the epimastigote form of the life cycle (Tyler & Engman 2001).

Morphology in the Insect Host

Epimastigote

These replicative forms are generally found within the mid gut of the Reduviidae host and can be easily maintained in axenic cultures making them the most accessible of the life cycle for study. Upon ingestion of a blood meal, a pleomorphic population of trypomastigotes and amastigotes are ingested (Andrews *et al*. 1987; Ley *et al*. 1988) and transformation of the trypomastigotes and amastigotes occurs in the mid gut into a short, replicative form in the gut or into long, slender, non-replicative forms which travel to the hind gut and rectum (de Souza 2002). They are spindle

shaped and are 20 – 40 μm in length with a basket shaped kinetoplast located anteriorally to the round and central nucleus. Its flagella emerge from the cell body as in the trypomastigote but beginning almost halfway down the length of its body and so has a much shorter undulating membrane (Figure 1.8 C). Whilst in the mid gut of the invertebrate host, the epimastigotes divide by binary fission and are closely associated with the microvilli of the intestine, attaching to the intestinal cells through the flagella by means that have still to be determined (Kollien & Schaub 2000). During the process of replication, various stages of the life cycle can often be observed; with intermediate amastigote-like (sphaeromastigote) and trypomastigote-like cells appearing. Division and replication occurs in the mid gut but many parasites can be found attached to the waxy cuticle lining of the insects' rectum. Whilst here the parasite is non-replicative and transforms into a metacyclic trypomastigote.

Metacyclic Trypomastigote

Attachment of epimastigotes to the cuticle of the rectum is a requirement for metacyclogenesis. This attachment is via non-specific hydrophobic interaction of the flagella and the waxy cuticle (Kollien & Schaub 2000). In experiments where interference with epimastigote attachment to culture vessels *in vitro* can prevent this transformation (Bonaldo *et al*. 1988) and enhancing the conditions required for attachment (hydrophobic, wax-coated culture vessels) can increase metacyclogenesis (Kleffmann, Schmidt and Schaub 1998).

The metacyclic trypomastigote is identical to the trypomastigote in morphology. Upon their generation in the Reduviid host they detach from the rectal gland and are expelled in the faeces and urine during a blood meal to enter the vertebrate host through mucosal membranes or the contamination of the feeding wound by infested faeces/urine. This latter method of infection usually occurs when the 'itchy' bite wound is scratched by the host and the infested faeces are accidentally rubbed into the wound due to their close proximity to the wound.

Life Cycle

The sylvatic cycle starts when the haematophagous invertebrate host feeds on the vertebrate host by sucking blood and ingesting the trypomastigote and amastigote forms of the parasite (Figure 1.9). There are approximately 110 species of triatomine bugs identified from the Reduviidae family that are potential vectors of *T. cruzi* (Garcia & Azambuja 1991). Of these, only 5 are of epidemiological importance: *Triatoma infestans, T. brasiliensis, T. dimidiate, Rhodnius prolixus and Panstrongylus megistus* (WHO 2002). Upon ingestion, the bloodstream trypomastigotes transform into epimastigotes and these, plus the ingested amastigotes, divide by binary fission in the intestine of the Reduviid bug and in the rectum, a certain proportion of the epimastigotes transform into metacyclic trypomastigotes which are eliminated with the faeces and are able to infect the vertebrate host (domestic cycle) (Garcia & Azambuja 1991; Kollien & Schaub 2000).

Contrary to what is known about African trypanosomes, the trypomastigote form of *T. cruzi* does not divide in the bloodstream and when introduced into the mammalian host, the metacyclic trypomastigote must invade a cell to complete its life cycle.

Unlike other intracellular parasites, *T. cruzi* invasion of the vertebrate host cell does not appear to involve an actin-mediated, phagocytosis-like process. Invasion appears to be initiated by parasite signalling causing the recruitment of host cell lysosomes (through Ca^{2+} regulated lysosome exocytosis) and their fusion at the host cell plasma membrane opposed to the site of parasite attachment allowing a gradual entry of the parasite through a host membrane derived lysosomal vacuole (Andrews 1995; Burleigh & Woolsey 2002; Ley *et al*. 1990; Rodriguez *et al*. 1996; Tan & Andrews 2002; Tardieux *et al*. 1992). This lysosomal-parasitophorous vacuole with its inherent acidic environment is essential for the subsequent differentiation of the trypomastigote into the replicative amastigote form and also to trigger the activation of a parasite derived pore-forming protein (TcTox) required for the eventual disruption of the vacuole (Andrews *et al*. 1990; Burleigh & Woolsey 2002; Tomlinson *et al*. 1995; Tyler & Engman 2001).

Transition of the trypomastigote to the amastigote in the cell begins in the parasitophorous vacuole with the transformation into a sphaeromastigote presenting a rounded shape with a long flagellum. However, after some hours, this flagellum has shortened, reaching a length of about 1 μm (de Souza 2002). During the trypomastigote-sphaeromastigote transformation there is an intermediary phase which is designated as epimastigote, although this form is somewhat different from the epimastigote observed in axenic cultures and the Reduviid mid gut. The sphaeromastigote form remains unchanged, increasing in size for a time of 20 – 35 h before initiating a process of binary division that is repeated every 12 – 14 h for around 9 cycles of division. When the cell is saturated with parasites, amastigotes begin their differentiation into trypomastigotes, a process that takes several h. Changes occur in the general organisation of the parasite at this point with a lengthening of the flagellum from 1 – 20 μm and the kinetoplast changing from its tighter, more spindle-like shape to a rounded shape with a loose basket arrangement of the k-DNA. The population of intracellular parasites does not differentiate completely synchronously and, therefore, at any given time all transitional stages between sphaeromastigotes, amastigotes, epimastigotes and trypomastigotes can be found in one cell. The intense movements of trypomastigotes at the end of this intracellular cycle disrupt the cell, releasing free parasites that can invade other cells. Cyst-like pockets of parasites (pseudocysts) can occur in various tissues such as cardiac muscle or brain tissue. If the arthropod vector takes a blood meal from the mammalian host at this time point of the life cycle it becomes infected with parasites. The vector will eventually require feed again, which will lead to the infection of the mammalian, thus propagating the infection.

Clinical Manifestations in Humans

Infection by *T. cruzi* in humans can cause a 2 stage disease: an acute stage occurring soon after infection lasting up to 3 months and a chronic stage that may not become symptomatic for 10 years after infection and can last the lifetime of the host.

Acute phase

An intense and localised inflammatory response occurs at the site of inoculation forming a small red nodule/lesion (chagoma). Where the infection has occurred through the conjunctiva of the eye, the inflammatory response causes oedema of the conjunctiva and eyelid and a swelling of the preauricular lymph node. These ocular symptoms collectively constitute the classical sign of acute infection known as Romaña's sign.

The acute phase is usually asymptomatic, but can present with manifestations that include fever, anorexia, lymphadenopathy, mild hepatosplenomegaly and myocarditis. Most acute cases resolve over a period of 2 to 3 months into an asymptomatic chronic stage.

During the acute phase there is an intense parasitaemia due to the cycle of intracellular infection and pseudocyst formation with the re-current rupture of cells by escaping trypomastigotes leading to further acute, local inflammatory responses accompanied by the further degeneration and necrosis of cells in the vicinity, particularly ganglion cells. Pseudocysts can be found in almost any tissue type and organ with the heart muscle usually invaded with some denervation occurring in this site (Marcelino de Souza *et al*. 2001; Rodrigues *et al*. 2002; WHO 2002).

Chronic stage

The chronic phase begins when the intense parasitaemia declines and the general symptoms of the acute infection disappear along with any other clinical symptoms of acute myocarditis. It is thought that equilibrium between the patients' immune responses and the parasite is reached with this status quo often remaining for the lifetime of the individual. Parasites can often be found circulating in the peripheral blood for many years after initial infection as well as IgG antibodies against *T. cruzi*. This latent period of infection (indeterminate form) affects around 50 – 70% of all infected individuals and lasts for the lifetime of the host. During this stage they are potential reservoirs of infection in endemic areas through Reduviid bugs and blood donation.

The symptomatic chronic stage may not occur for years or even decades after initial infection and affects 30 – 50% of infected individuals. These patients are often aged 20 – 50 years old (an economically productive group). Its manifestations include cardiomyopathy (the most serious manifestation), pathologies of the digestive tract such as megaesophagus and megacolon and weight loss. Chronic Chagas disease and its complications can be fatal with most deaths occurring due to heart failure.

Treatment

If the symptoms are recognised in the acute phase, effective chemotherapy is available in the form of nitroimidazole or nitrofuran derivatives such as benznidazole or nifurtimox. However, resistance to these drugs are being reported (Buckner *et al*. 1998). Both drugs require a high degree of patient compliance in order for optimum results. Nifurtimox is given at a dose of 5 mg/kg/day orally slowly increased to 15 mg/kg/day (divided over 3 doses) for 2 to 4 months during the acute cycle of infection. However, this drug is now no longer being produced and, whilst there are still stocks available, the cost to the patient can be prohibitive ($48 US per treatment regimen, the equivalent of one month's pay for a Bolivian miner; (Weir 2006). Benznidazole is generally given at 5 – 10 mg/kg/day orally in 2 daily doses for 1 – 2 months. Side effects are most common in adults for both drugs with a range of symptoms common to both, varying from neurotoxicity, anorexia, nausea and leukopoenia with nifurtimox exhibiting these most frequently and benznidazole showing a greater degree of skin manifestations such as rashes, occurring in as much as 30% of patients (Castro, de Mecca and Bartel 2006). This drug is no longer produced by the original manufacturer (Roche) and is now produced by the Brazilian government who were given the commercial rights and equipment to manufacture this drug. Problems with the supply of the primary constituent of this drug have lead to delays in its distribution, often up to 4 months (Weir 2006).

One of the favoured drugs currently being used is benznidazole. Its use in acute infections is as an antiparasitical but its use in chronic infection is regarded as controversial. It is now being used in children as a treatment for chronic infection due to the discovery that chronic symptoms are not due to autoimmune disease but

inflammatory reactions induced by the continual release of parasites at local lesions. When administered to serologically positive school children (7 – 12 years old) chronically infected, benznidazole converted 60% of the children to sero-negativity with no further development of heart damage (Sgambatti de Andrade *et al*. 1996; Sosa Estani *et al*. 1998). In murine studies of chronic infection, Benznidazole administration can also lead to a reduction in cardiomyopathy (Bustamante *et al*. 2007) and further clinical studies in adults also found reduced cardiomyopathy and increased sero-negativity with drug treatment (Viotti *et al*. 2006).

However, in chronic infection the most frequently used treatment is the management of the clinical manifestations of the disease such as pace makers being fitted for heart arrhythmias and chronic heart failure, transplantation and surgery for megacolon.

Increasing drug resistance and reduced drug availability is leading to advances in the search for alternative chemotherapies for this infection. One component is the search for plant metabolites and plant extracts as a source of new antiparasitical drugs. Naphthylisoquinoline alkaloids from tropical plants have wide ranging parasiticidal activities affecting *Plasmodium* (Bringmann *et al*. 2003a; Francois *et al*. 1997), *Leishmania* (Bringmann *et al*. 2000; Bringmann *et al*. 2003b) and *Trypanosoma* spp. (Bringmann *et al*. 2008). Unfortunately the plants from which the alkaloids are derived are difficult to cultivate and are rare. However efforts are being made to synthesise these compounds and promising results have been achieved with the synthesised compounds being as effective as their natural counterparts (Bringmann *et al*. 2008). Other alkaloids are also effective, with the canthinones exhibiting effectiveness in the acute stage of infection in mice and short-term chronic stage, allowing 80 – 100% survival compared to control groups, at a dosage and effectiveness comparable to that of Benznidazole (50 mg/kg/day) (Ferreira *et al*. 2007).

Triterpenic compounds also have antitrypanosomal effects on trypomastigotes isolated from infected mice (Rosas *et al*. 2007) and another class of terpenes isolated

from plants, the sesquiterpenes affect the growth of epimastigotes *in vitro* (Brengio *et al*. 2000) as do sesquiterpene lactones (Jimenez-Ortiz *et al*. 2005).

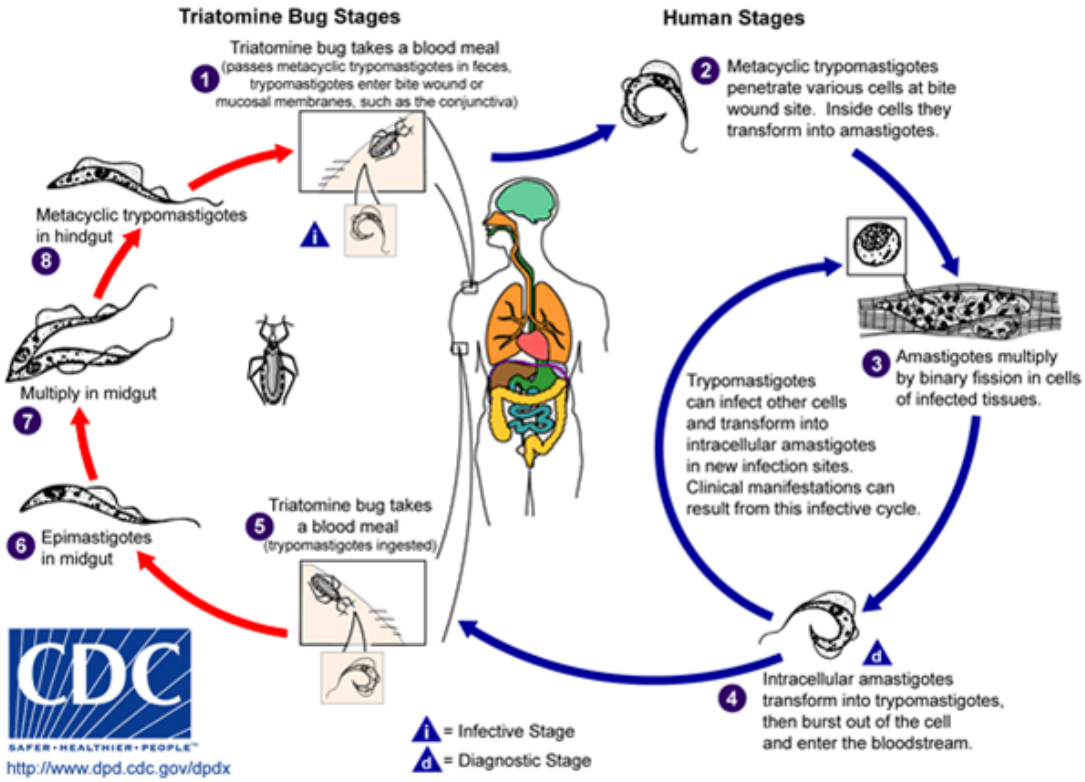


Figure 1.9 Life-cycle of *T. cruzi*.
The life cycle is initiated when an infected triatomine insect vector (Reduviidae) takes a blood meal and releases metacyclic trypomastigotes in its faeces or urine near the site of the bite wound. Scratching and rubbing the irritated bite wound allows entry of these trypomastigotes to the host through the wound itself or through intact mucosal membranes, such as the conjunctiva❶.

Inside the host, the trypomastigotes invade cells, where they differentiate into intracellular amastigotes within a parasitophorous vacuole❷. The amastigotes multiply by binary fission ❸ and differentiate into trypomastigotes and then are released into the circulation as bloodstream trypomastigotes❹. Trypomastigotes infect cells from a variety of tissues and transform into intracellular amastigotes in new infection sites. Clinical manifestations can result from this infective cycle. The bloodstream trypomastigotes do not replicate (different from the African trypanosomes). Replication resumes only when the parasites enter another cell or are ingested by another vector. The "kissing" or "assassin" bug becomes infected by feeding on human or animal blood that contains circulating parasites❺. The ingested trypomastigotes transform into epimastigotes in the vector's midgut❻. The parasites multiply and differentiate in the midgut ❼and differentiate into infective metacyclic trypomastigotes in the hindgut❽. *Trypanosoma cruzi* can also be transmitted through blood transfusions, organ transplantation, transplacentally and in laboratory accidents. Source: Figure from the CDC.

1:8 Research Hypothesis

Little scientific work has been carried out on plant essential oils (PEOs) and their potential use as a source of novel antiparasitical drugs. Whilst there is much evidence for plant extracts (as obtained through alcoholic extractions of plant material) having antimicrobial, antifungal and antiviral action, with a brief search in PubMed showing over 3000 articles (accessed 25th June 2007) a similar enquiry for plant extracts with coccidian, *Giardia* and *Trypanosoma* parasites found only 214 articles with only 8 of them concerned with PEOs and all within the past 10 – 15 years. This search is broken down in the following way:

- 16 articles for plants with action against coccidian parasites and 33 against *Giardia*. Of these papers, 5 were concerned with PEOs that had anticoccidial and anti-flagellate effects. These 5 papers covered 7 PEOs and a total of 9 parasites with 2 of the PEOs being found to have anti-giardial activity. There was nothing found for plant oils against *Cryptosporidium*.

- 165 articles for plants tested for antitrypanosomal action. Of these, only 3 articles concerned the antitrypanosomal effect of PEOs for a total of 5 PEOs and 2 species of *Trypanosoma*.

From this it can be readily seen that there is a dearth of relevant investigative data concerning PEOs and their possible anti-parasitical actions. The few PEOs that have been investigated have shown great promise in their antiparasitical properties with their active constituent being isolated and often found to have greater effect than the whole oil and also more effective than the drug of choice. However, with less than 10% of approximately 250,000 of the world's flowering plant species having been tested and examined scientifically for their medicinal properties (Editorial 1994), the few PEOs already reported is just the tip of the iceberg.

As *Giardia* and *Cryptosporidium* have a worldwide distribution and infect millions of people each year (an estimated 280 million for *Giardia* alone) and *Trypanosoma cruzi* is responsible for around 50,000 deaths and 16 – 18 million infections *per*

annum in South America, but with little effective chemotherapy available, there is a real need for new treatments that are cheap, readily available and easy to produce. Plants and their extracts may, in endemic areas, be the only accessible form of treatment and so the traditional uses of certain plants must be preserved and scientifically examined for potentially new drugs.

Much of our current knowledge of the use of plants has come through traditional remedies. In the United Kingdom an historical use of the Bilberry (syn. Whortleberry, Blueberry, Blaeberry and Whinberry) is shown in the following passage:

> "…sell us whortle berries, or the vaccinia nigra of Virgil,… …the astringency lies all in the black skin and not in the pulse. Our soldiers eat them for the bloody flux, while encamped at Fort-Angus (Fort Augustus – Ed.)…our men were getting flux from loch [Ness] water…" – Anon. Physician, 3rd September 1746. (Anon. 1825)

The true causes of such fluxes (diarrhoea) are poorly understood, but could be due to various enteropathogens including *Giardia* and *Cryptosporidium*.

Other evidence for the treatment of fluxes using bilberries comes from the study of archaeological material at microscopic level. An archaeobotanical / archaeomedical project investigating the contents of a sealed hospital drain dating from the early 14th century AD (Soutra) in Scotland, UK discovered *Giardia* cyst remains in human faecal material in the presence of bilberries. Also identified were *Ascaris* and *Trichuris* ova in the presence of common tormentil (*Potentilla erecta*) and wild strawberry (*Fragaria vesca*) (Personal Communications, Dr. Brian Moffat, Director of the Soutra Hospital Archaeoethnopharmacological Research Project [SHARP], Edinburgh, 2006). The Augustinian canons at that hospital and other sites used these plants as remedies for helminthic infections and bilberries, in particular, for diarrhoea.

Due to such evidence being previously discovered at Soutra, investigations which would identify other enteric protozoan parasites were initiated with the hope of being

able to identify them molecularly as well as microscopically and, if possible, to identify plant material which may have been used to treat the parasite infection.

Such evidence suggests that there is a potential not only for PEOs having antiparasitical actions but also the fruits of some plants might also be a source of new drugs. Also with the current trend for 'functional foods', that is to say foods which have more than just a nutritional value and have other 'health benefits' such as influence over the immune system or reduction of cholesterol, the consumption of fruits when diagnosed with a parasitic illness may be an effective (and tasty) alternative to conventional chemotherapy with reduced occurrences of side effects, increased patient compliance and a potential source of novel drugs. The research hypothesis for this project is that plant products have the potential to inhibit protozoan parasite of medicinal importance.

The rather specific number of PEOs and polyphenol-rich fruit extracts (PRFEs) studied reflects merely the availability of the material at the commencement of study due to their costs and availability. PEOs were gifted by the essential oil manufacturer (F.D. Copeland and Sons) and the PRFEs gifted by the Scottish Crop Research Institute.

1:9 Project Aim and specific objectives

This project intends to investigate 12 different PEOs and 11 different PRFEs for activity against the protozoan parasites *G. duodenalis, C. parvum* and *T. cruzi*. The aim of the project is to determine potentially active compounds from the composition of the effective PEOs and their mechanism of action through microscopic and biochemical means. The specific objectives of this project are:

- To assess the current scientific literature for the antiparasitical effects of PEOs and to identify areas of further study.
- To determine the minimum concentration of PEOs required to inhibit the growth of *G. duodenalis* trophozoites and *T. cruzi* epimastigotes and to influence the excystation of *C. parvum* oocysts.

- To determine the minimum concentration of PRFEs required to inhibit the growth of *G. duodenalis* trophozoites and *T. cruzi* epimastigotes and to influence the excystation of *C. parvum* oocysts.
- To determine which factors such as temperature, time, pH, bile modify or alter the activity of the above products.
- To determine the mode of action of the above products by investigating their effects on motility, morphology and biochemistry on the parasites mentioned.
- To compare the inhibitory properties of the PEOs and PRFEs with drugs currently used for the treatment of infections caused by the above parasites.
- To determine the validity of the historical / traditional use of bilberries for the treatment diarrhoea by investigating the *in vitro* inhibitory properties of extracts of this fruit upon *G. duodenalis* trophozoites and *C. parvum* oocysts.
- To determine the presence of plant material in conjunction with protozoan parasites in archaeological samples and to assess their use as medicinal herbal remedies from the Viking to the medieval period of British history.
- To identify protozoan parasites within archaeological samples from Viking latrines and medieval hospital drains using microscopic (fluorescent and bright field) and genetic methods.

CHAPTER 2

Giardia duodenalis

The inhibitory effect of various PEOs and their constituents on *Giardia duodenalis* trophozoite viability *in vitro*.

2:1 ABSTRACT

The effects of a range of plant essential oils (PEOs) on *G. duodenalis* trophozoite viability *in vitro* were investigated in this study. All PEOs tested demonstrated antigiardial activity at a concentration of 0.02% (200 μg ml^{-1}) and in a dose dependent manner, with oils of myrrh, myrtle and elemi having the greatest inhibitory effect with a minimum inhibitory concentration (MIC) of 0.005% (50 μg ml^{-1}) respectively which was more effective than metronidazole (MIC = 67 μg ml^{-1}). Metronidazole had a greater effect on trophozoite viability at lower concentrations than any of the oils tested where the 90% and 50% inhibitory concentrations (IC_{90} and IC_{50}) were 21.8 μg ml^{-1} and 5.6 μg ml^{-1}, respectively. All the PEOs tested failed to reach these levels and had a wide range of activity (IC_{90} = 0.003 – 0.013 % [30 – 130 μg ml^{-1}], IC_{50} = 0.0009 – 0.007 % [9 – 70 μg ml^{-1}]). All the PEOs, with the exception of myrrh were able to reduce trophozoite viability to 0% within 60 min of incubation at 0.005% (50 μg ml^{-1}). Both myrtle and elemi inhibited the expression of a 37 kDa protein in whole cell lysates which was also observed in trophozoites killed using either metronidazole or by flash freezing in liquid nitrogen. Heat killed trophozoites failed to abolish this protein. Myrtle oil also inhibited a ~70 kDa protein and elemi upregulated a 100 kDa protein in a concentration dependent manner. The major constituents of myrtle (α-pinene and eucalyptol) and elemi (limonene), when used at concentrations equivalent to that found in 0.02% of whole oil, were able to kill all trophozoites, but not at lower concentrations. Also, these compounds did not have an effect on the reported actions on protein expression, suggesting that the compounds present in the greatest proportions are not necessarily responsible for the reported activity. To conclude, PEOs can kill *G. duodenalis* trophozoites and modify the morphology of *G. duodenalis*. Terpenic compounds present in these oils may be responsible for this activity. The rapid action of PEOs has shown a potential for novel drug design which could reduce the duration of treatment. Furthermore, myrtle and elemi oils can interfere with the protein profile of *G. duodenalis* trophozoites as well as reduce their viability.

2:2 INTRODUCTION

The protozoan parasite, *Giardia duodenalis* (syn. *G. lamblia*, *G. intestinalis*) has a worldwide distribution and is a common cause of diarrhoea. Whilst there is effective drug treatment available for *G. duodenalis*, anecdotal evidence of resistance has been shown (Wright *et al*. 2003). Oral metronidazole is the drug of choice, but side effects such as nausea, headaches and an unpalatable strong metallic taste reduce patient compliance.

G. duodenalis has been recognised as an anaerobe although recent evidence suggests that this organism does require very small amounts of oxygen to survive (Bénéré *et al*. 2007). This can usually be provided by their axenic culture in tubes which are filled to 90 – 95% of their capacity. Traditionally 2 major choices for susceptibility assays have been available: tube assays and microtitre plate assays. Microtitre plates are problematic due to the variability of the anaerobic environment and the need to remove the plates from the anaerobic or low-oxygen environment to monitor the progress of the assay. Tube assays are cumbersome and time-consuming, especially when trying to screen many novel drugs, with few if any replicates possible (Upcroft & Upcroft 2001). The use of adhesive sealant tape to cover the wells of the microtitre plate has alleviated the problems of low-oxygen environments whereby filling the wells of the plate to almost their maximum capacity and sealing in a small air bubble creates a micro-environment suitable for trophozoite survival (Bénéré, 2007).

Population densities of *Giardia* can have an impact on their culture and on susceptibility assays. Using few trophozoites, it becomes difficult to determine viability using chemical assays such as tetrazolium salt reduction and by enumeration with microscopy and Trypan blue inclusion / exclusion. The same problems arise when the population density is too great. When performing susceptibility assays it is necessary to use a population density such that an accurate measurement can be taken of trophozoite viability whether it is a concentration required to inhibit the viability of 50% of trophozoites or their complete inhibition. A seeding density previously used by Upcroft & Upcroft (2001) of 4×10^4 trophozoites per well of a 96 well microtitre plate was found to be sufficient for a visual assessment of viability

after 72 h. In [^{3}H] thymidine assays initial inoculates of 10^3 per well have been successfully used from 24 h to 96 h of incubation (Perez *et al*. 2001). Biochemical reduction of tetrazolium salts has been found to be accurate when using 5 x 10^4 trophozoites per well for 72 h incubation (Bénéré 2007). As can be seen, there is a range of trophozoite concentrations that can be used, dependant on the assay used to assess viability.

The solubility of drugs can be problematic when trying to assess their effectiveness *in vitro* and they are usually used in conjunction with diluents. In the case of the plant essential oils to be used herein, solubility is of major importance as is the potential effects of the diluent used to solubilise the oils. Common diluents used with *in vitro* experiments are ethanol and dimethyl sulphoxide (DMSO). Both can have adverse effects on the viability on *Giardia* trophozoites when used at high concentrations (70% for ethanol; Freitas *et al*. 2006 and 0.05% for DMSO; Campanati, Gadelha and Monteiro-Leal (2001) although DMSO is used as a cryopreservative at concentrations of up to 10%; Phillips, Boreham and Shepard (1984). As the maximum concentration of diluent to be used *in vitro* will be 0.06%, the effects of each diluent must be assessed at this concentration and above.

The nitroimidazole, metronidazole, is the drug of choice for giardiasis (Upcroft & Upcroft 1993), but with variability of its *in vitro* effects being reported (2.5 µM [Bénéré 2007] – 6.3 µM [Upcroft & Upcroft 2001]). The reduction of metronidazole resistance in resistant strains has also been reported when these strains are cultured in the absence of the drug for long periods of time (Upcroft & Upcroft 2001). Therefore, it is important to be able to verify a working concentration of drug that can kill 100% of trophozoites (minimum inhibitory concentration; MIC) within the time frame of the experiment. To this end, a titration of metronidazole was carried out to calculate the MIC of the drug.

Recent advances in the understanding of the biochemistry and molecular biology of protozoa have permitted the study of molecules that directly participate in the parasites' life cycle or in pathogenicity (North, Mottram and Coombs 1990). For

example, the understanding of antigenic variance can allow inferences to be made on the survival strategies used by the parasite (Müller & Gottstein 1998) and vaccination against the parasite (Olson, Ceri and Morck 2000). Antibiotic resistance can be characterised and the mechanisms by which the parasite can achieve resistance can be investigated (Müller *et al*. 2007; Upcroft & Upcroft 2001; Upcroft *et al*. 1996).

Plant oils and extracts made from them have a wide range of effects on cell biology whether it is on the membrane, interfering with surface proteins, or within the cell, inhibiting proteins.

The method used for primary protein analysis is sodium dodecyl-polyacrylamide gel electrophoresis (SDS-PAGE). One-dimensional SDS-PAGE or 2-dimensional gel electrophoresis (2DE) are commonly used techniques for resolving protein solutions for further analysis. This technique separates proteins according to molecular weight and electrophoretic mobility and can be used to discover differences in protein quantities / profiles between various samples.

As antibiotic resistance has been noted in *Giardia* new drugs are being sought to combat the infection (Upcroft & Upcroft 2001). The antimicrobial properties of plant products have been recognised for numerous years and recently there has been renewed interest with plants and plant products being used as traditional remedies for various ailments in numerous countries, with little or no side effects (Jones 1996; Heinrich 2000 & 2003). Plants can have inhibitory properties against a range of microbes such as *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Candida albicans* with researchers investigating the historical and folk lore uses of plants in different parts of the world such as South America (Rosado-Vallado *et al*. 2000; Yasunaka *et al*. 2005), India (Samy, Ignacimuthu and Sen 1998; Samy & Ignacimuthu 2000), Africa (Kudi *et al*. 1999) and the Middle East (Essawi & Srour 2000). Even the antiviral properties of plants are being investigated (Cowan 1999; Jassim & Naji 2003). The antimicrobial properties of herbs and spices have been recognised for thousands of years and recently there has been renewed interest in

such products, especially plant oils (Smith-Palmer, Stewart and Fyfe 1998; Kumar & Berwal 1998).

An ethanol extract of clove oil was found to inhibit prostaglandin E_2 (PGE_2) production in activated murine macrophages (Kim *et al*. 2003) and 1,8-cineol from eucalyptus oil inhibited cytokine production (Juergens *et al*. 2003). It is thought that the cytopathic effects of TTO is due to its lipophilic character, with the oil interacting with the cellular membrane and disrupting normal membrane activity (Soderberg, Johansson and Gref 1996). Antibody led responses can also be affected by oils with rosmarinic acid from lemon balm inhibiting the classical complement pathway by blocking both C5 convertase (Peake *et al*. 1991) and C3 convertase (Englberger *et al*. 1988). The inhibition of PGE_2 production in activated murine macrophages by eugenol, prepared from an ethanol extract from cloves, is due to the inhibition of COX-2 mRNA expression (Kim *et al*. 2003). In rats, the fixed oil of basil was found to inhibit both cyclooxygenase and lipooxygenase pathways of arachidonic acid metabolism (Singh 1999). The mode of action of allicin and its condensation product, ajoene, have both been investigated and it has been reported that the mechanism of action is through interaction with important thiol-containing enzymes (Ankri & Mirelman 1999). Ajoene can inhibit *T. cruzi* proliferation by possibly inhibiting phosphatidylcholine biosynthesis (Urbina *et al*. 1993).

Much of the work concerning plants has dealt with alcoholic extracts, but some investigations have been carried out using the concentrates produced by steam distillation of plant material. This is the essential oil which has common uses as fragrance in perfumes and as flavourings in food. These are refined extracts of plants, concentrating the many constituents into a lipophilic, low density formulation which may enhance the adsorption of active constituents from within the oil and enhance the targeting of active constituents within the oil to intracellular parasites (Boyom *et al.* 2003). Investigations have shown that plant oils can have antimicrobial/antifungal activity (Fyfe, Armstrong and Stewart 1997; Smith-Palmer, Stewart and Fyfe 1998, 2002; Hammer, Carson and Riley 1999; Burt 2004; Hammer, Carson and Riley 2003; Terzi *et al*. 2007; Kalemba & Kunicka 2003), antiviral effects (Cowan 1999;

Jassim & Naji 2003) and more recently, antiparasitic properties (reviewed by Anthony, Fyfe and Smith 2005).

Garlic extract inhibits *G. duodenalis* trophozoite growth *in vitro*, primarily due to the transformation product (diallyl trisulphide) of its major constituent (allicin) (Lun *et al*. 1994). Treatment of humans with garlic extract can reduce clinical signs and symptoms more rapidly than metronidazole, the drug of choice (Soffar & Mokhtar 1991). Whole garlic extract co-cultured with trophozoites causes internalisation of flagella and ventral disc fragmentation (Harris *et al*. 2000; Anthony, Fyfe and Smith 2005). Thyme oil can also be as effective as metronidazole in killing *G. duodenalis* cysts (Sahebani, Farsangi and Movahed 2004). More recently, lavender oil from *Lavandula angustifolia* and *L. intermedia* were found to inhibit the growth of *G. duodenalis* trophozoites *in vitro* at concentrations of < 1% (Moon, Wilkinson and Cavanagh 2006).

There is limited information available for the antigiardial properties of plants. A brief search on PubMed shows 35 journal articles for plants with antigiardial properties, most concerning the crude extract of the plant or alcoholic extracts (PubMed search dated 26th Sept. 2007). Of these articles, only 2 investigated the antigiardial properties of plant essential oils (thyme and lavender oil). This leaves our understanding of PEOs as a potential source of novel antigiardial drugs as scanty, at best. As PEOs are a concentrate of various constituents, the identification of active compounds from either the crude extracts or the more refined extractions can assist in targeting future novel drug searches. A range of compounds are already known to inhibit the growth or kill *G. duodenalis* trophozoites both *in vitro* and *in vivo*, including the flavones (Khan *et al*. 2000), flavenoids (Barbosa, Calzada and Campos 2006 & 2007; Calzada, Cervantes-Martínez and Yépez-Mulia 2005; Calzada *et al*. 2003; Calzada, Meckes and Cedillo-Rivera 1999), flavenol glycosides (Calzada & Alanís 2007; Calzada 2005), alkaloids (Moo-Puc *et al*. 2007), terpenoids (mono, tri and sesqui) (Mena-Rejón *et al*. 2007; Said Fernández *et al*. 2005), gallic acids (Alanís *et al*. 2003) and proanthocyanidins (Calzada *et al*. 2001; 1999).

In this series of experiments the effect of incubating 12 different PEOs over a 24 h period with intact *G. duodenalis* trophozoites to determine whether they influenced *G. duodenalis* trophozoite viability *in vitro* was investigated. The minimum inhibitory concentrations for these PEOs were also determined and their active constituents discovered. Further, an analysis into the possible mechanism of action of the effective oils and constituents were performed using 1-D SDS-PAGE fractionation.

2:3 MATERIALS AND METHODS

2.3.1 Parasite culture

Source and maintenance of G. duodenalis trophozoites

Trophozoites of the BVM strain of *G. duodenalis* (donated by Dr. T. Paget, The Universities of Kent and Greenwich at Medway, UK) were maintained axenically in sealed flat sided 110 mm x 16 mm culture tubes (Nunc, Denmark) angled 5° from the horizontal, at 37ºC, in a modified TYI-S-33 medium (Appendix 4) supplemented with 10% (v/v) heat-inactivated foetal bovine serum (Gibco-BRL, Paisley, UK) and filled to maximum capacity. Sub-culturing was performed routinely at 72 – 96 h intervals by harvesting from the culture tubes through chilling on ice water (to facilitate trophozoite detachment) for 20 min and then pipetting 1 ml of the suspended trophozoites into ~9 ml of fresh TYI-S-33 medium.

G. duodenalis enumeration for experiments

Trophozoite enumeration was performed using an improved Neubauer haemocytometer and trophozoite viability determined by Trypan blue inclusion / exclusion where dead cells (stained cells) were determined microscopically. A lack of motility and changes to the morphology served as corroborating parameters of cell death (TBMM). Briefly, trophozoites were harvested from the culture tubes following chilling on ice water for 20 min and then centrifuging the culture tubes (500 x ***g*** for 5 min). The supernatant was aspirated to waste and the pellet resuspended in 1 ml TYI-S-33 medium. A 20 µl aliquot of this suspension was added to 80 µl of TYI-S-33 medium in a 1.5 ml microcentrifuge tube to dilute the numbers of trophozoites for easier counting. A 20 µl aliquot of this suspension was then added to 20 µl 0.4% Trypan blue solution (Gibco, UK) and mixed thoroughly. After mixing, 10 µl aliquots were dispensed into both chambers of an improved Neubauer haemocytometer, allowed to settle (30 – 60 sec) then enumerated. Trophozoite enumeration was determined under bright field microscopy with trophozoites being counted within the grids of the haemocytometer chamber. The average number of live trophozoites from each chamber counted in five large grids was used to enumerate the sample and calculated as follows:

Average number of trophozoites x dilution factor x 5 x 10^4 = Trophozoites ml^{-1}

Viable trophozoites were determined by Trypan blue exclusion, motility and flagellar movement. Trophozoites were determined to be dead following the inclusion of Trypan blue and/or a lack of motility.

G. duodenalis titration for in vitro experiments

Initial experiments used flat sided 110 mm x 16 mm culture tubes containing 1 x 10^6 trophozoites ml^{-1}, however, it was soon discovered that the parasites could be cultured for the duration of an experiment in 96 well microtitre plates. This allowed for a more rapid screening of PEOs. A concentration of trophozoites that would provide an even monolayer of trophozoites in microtitre plate wells was determined. Trophozoites at concentrations ranging from 3.3 x 10^4 – 3.3 x 10^5 trophozoites ml^{-1} were assessed over 24 h at 37°C using 150 µl $well^{-1}$ and 150 µl of modified TYI-S-33 medium. A concentration of 2.7 x 10^5 trophozoites ml^{-1} (equivalent of 4.5 x10^4 $well^{-1}$) was found to give an even monolayer over 24 h and was used for experiments in 96 well microtitre plates (data not shown).

Parasite lysate preparation

Parasite concentrations of 2.7 x 10^5 ml^{-1} to 5 x 10^7 ml^{-1} were prepared in 1 ml volumes of complete TYI-33-S medium initially and then treated to three cycles of centrifugation (500 x ***g***, 5 min) and washing in 1 ml volumes of electrode running buffer (TGS). The supernatant was aspirated to waste and the pellet resuspended in 100 µl of TGS and treated to 15 cycles of freeze / thawing in liquid nitrogen (1 min) and 56°C (1 min). This lysate was then centrifuged (12,500 x ***g***, 1 min) and the resultant supernatant removed to a fresh 1.5 ml microcentrifuge tube and kept at -80°C until used in electrophoresis.

2:3.2 Experimental treatments of parasites

Ethanol and dimethyl sulphoxide titrations

To determine which diluent could be safely used in the experiments a titration was carried out using 2 potential diluents (Ethanol [EtOH] and dimethyl sulphoxide [DMSO]) from a starting concentration of 75% diluent / 25% distilled water. A final

concentration equivalent to 0.02% v/v in the microtitre well was achieved by diluting 10.67 µl of diluent (75% v/v stock EtOH or DMSO) in 1 ml of modified TYI-S-33 medium and vortexing for 20 sec. 150 µl of this solution was then immediately added to 150 µl of 2.7 x 10^5 trophozoites ml^{-1}. Doubling dilutions of the solution were performed to give a range of concentrations equivalent to those used in PEO titrations (0, 0.0067, 0.0167, 0.033, 0.067% EtOH or DMSO v/v). Incubation occurred in sealed 96 well microtitre plates at 37°C for 24 h following which parasite viability was assessed by TBMM. The diluent which failed to have any significant detrimental impact on trophozoite viability was used as the diluent of choice for PEO experiments.

Incubation of G. duodenalis trophozoites with metronidazole

A concentration of 2.7 x 10^5 trophozoites ml^{-1} was tested against the reference drug metronidazole at the following concentrations: 0, 1.7, 2.5, 3.35, 6.7, 16.7, 33.5, 50.2, 67, 83.7 and 167 µg ml^{-1} diluted in TYI-S-33 medium with 150 µl of each concentrate being added to 150 µl of trophozoites. Incubation occurred in sealed 96 well microtitre plates at 37°C for 24 h. Following titration (Figure 2.2), metronidazole at a concentration of 67 µg ml^{-1} (20 µg per 300 µl well) was determined to be the minimum concentration that killed 100% of trophozoites *in vitro*. This concentration was used in all positive controls.

PEOs preparation

All PEOs were diluted in absolute ethanol to provide 25% v/v stock solutions (25% PEO, 75% absolute ethanol), which were used in these experiments. The full list of PEOs investigated is given in Appendix 1. A final concentration of 0.02% in the microtitre well was achieved by diluting 10.67 µl of PEO (25% v/v stock) in 1 ml of modified TYI-S-33 medium and vortexing for 20 sec with 150 µl of this being immediately added to 150 µl of 2.7 x 10^5 trophozoites ml^{-1}. Thus, the maximum concentration of ethanol in the well was 0.067%, which has no effect on the viability of trophozoites during the incubation period used.

Incubation of G. duodenalis trophozoites with PEOs

After 72 h of culture, trophozoites were harvested by chilling culture vessels in iced water for 20 min. Experiments were performed in sterile, 96 well microtitre plates covered with plate sealer film and lids (Costar, Corning). Final concentrations of 0.02% of PEOs were initially used. Where 100% killing of trophozoites was observed, the PEOs responsible were further titrated at concentrations of 0.01, 0.005, 0.0025 and 0.00125% using doubling dilutions to determine their MIC. The microtitre plates were sealed and incubated for 24 h at 37ºC and after 2 h of incubation the parasites were examined microscopically in their wells for adherence and, also again at 24 h, prior to the determination of viability with Trypan blue.

The positive control consisted of 4 x 10^4 trophozoites in 300 µl TYI-S-33 containing 67 µg ml^{-1} of metronidazole which had been previously assessed as the MIC for this drug with this concentration of trophozoites, isolate and method. The negative controls consisted of 4 x 10^4 trophozoites in 300 µl TYI-S-33 in the absence of PEOs or metronidazole. All samples were prepared in triplicate.

Incubation of G. duodenalis trophozoites with PEO constituents

Following on from the PEO titration experiments, three PEOs exhibited the greatest antigiardial properties as assessed by MIC. Oils of myrrh, myrtle and elemi were more closely examined for their composition using Gas Chromatography coupled with Mass Spectroscopy (GC-MS). This procedure was carried out by F.D. Copeland & Sons with the composition of the PEOs given in Appendix 3. Constituents which made the greater part of the PEO were assessed at their equivalent concentration within the PEO such that a constituent which constitutes 75% of the make up a PEO would be used at a concentration equal to 75% of the final concentration of the whole PEO, i.e. 75% of 0.2% = 0.15%. Where there were multiple constituents found in similar concentration within a PEO they would be used at their equivalent concentrations individually and in combination to determine the individual active constituent and if any effects observed were additive in action or were cumulative. In the case of myrtle oil the major constituents were found to be the monoterpenes α-pinene and eucalyptol (syn. 1,8-cineole) comprising 51.72% and 22.35% of the

whole oil respectively. Elemi oil composition was found to consist of the terpene limonene (syn. (+)-*p*-Mentha-1,8-diene; (+)-Carvene; (*R*)-4-Isopropenyl-1-methyl-1-cyclohexene) (51.54%), α-phellandrene (16.23%) and elemol (10.90%). Myrrh oil composition was found to consist of furanic sesquiterpenes furanodiene (31.97%) and curezene (26.69%). At the time of writing only α-pinene, eucalyptol and limonene were readily available from chemical companies and these were used in experiments and prepared in the same manner as their whole oils.

Incubation of G. duodenalis trophozoites with PEOs and PEO constituents over a 2 h time course

During the 2 h observation period for trophozoite adherence it was noted that there was evidence of antiparasitical action occurring already. A short time course experiment was employed to determine how quickly the PEOs exhibited antigiardial effects. *G. duodenalis* trophozoites were incubated in sealed microtitre plates for 30, 60 and 120 min in the presence or absence of PEOs and the constituents' α-pinene, eucalyptol and limonene at their MIC (Table 2.1).

Table 2.1 Minimal inhibitory concentration of PEOs used in experiments.

MIC (%)	PEO
0.02	Amyris
0.02	Buchu
0.02	Lavender
0.02	Patchouli
0.02	Sweet Fennel
0.01	Geranium
0.01	Marjoram
0.01	Palmarosa
0.01	Thyme
0.005	Elemi
0.005	Myrrh
0.005	Myrtle
0.002586	α-Pinene
0.002577	Limonene
0.00112	Eucalyptol

2:3.3 Parasite culture analysis

G. duodenalis cell count assay

Trophozoite enumeration was performed using an improved Neubauer haemocytometer and trophozoite viability determined by TBMM. Briefly, trophozoites were harvested from wells by chilling the plate on ice water for 20 min and the contents of triplicate wells were combined into 1.5 ml microcentrifuge tubes and then centrifuged (10 sec, bench top centrifuge 5410, 1.5 ml microcentrifuge tube GmbH, Germany). The supernatant was aspirated to waste and the pellet resuspended in 100 µl TYI-S-33 and 100 µl 0.4% w/v Trypan blue solution (Gibco, UK) and mixed thoroughly. After mixing, 10 µl were dispensed into both chambers of an improved Neubauer haemocytometer, allowed to settle (30 – 60 sec) then assessed. Trophozoite viability was determined under bright field microscopy using the following formula: (L ÷ (L+D)) x 100 where L is the number of live trophozoites (unstained, motile and altered morphology from pyriform) and D the number of dead trophozoites (stained with Trypan blue, non-motile,) at a total magnification of x400. In all cases the trophozoites were enumerated in batches where 4 sets of triplicate wells were harvested at any one time and Trypan blue exposure was always < 5 min for each sample with the remaining samples kept on ice until enumerated.

SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) analysis

Initial experiments were prepared to determine the appropriate numbers of parasites required for electrophoretic analysis. Concentrations of trophozoites used in these experiments ranged from 2.7 x 10^5 ml^{-1} to 5 x 10^7 ml^{-1} for each well of a 96 well microtitre plate in quintuplicate. From these results, a concentration of 5 x 10^7 ml^{-1} trophozoites would be required in experiments involving PEOs and their constituents. A Bio-Rad Protean 3 mini-prep system (Bio-Rad, USA) was employed with a 12% acrylamide separating gel and 4% acrylamide stacking gel prepared according to the manufacturer's instructions (Appendix 5).

10 µl aliquots of lysate prepared from 5 x 10^7 ml^{-1} trophozoites were added to 30 µl of sample buffer and heated to 100°C in a water bath for 5 min. To the first well of a 10 well SDS-PAGE gel were added 10 µl of molecular weight marker (Precision

Plus Protein standard; unstained; 10 – 250 kDa, Bio-Rad) and to each of the following wells were added 30 µl of the boiled lysate / sample buffer mix. This would give a maximum concentration of 3.75 x 10^6 parasites $lane^{-1}$. The optimal voltage and running time combination was determined to be at 180V and 55 min or 200V and 40 min for the best separation of proteins with 180V and 55 min being found to have the best resolution (data not shown). On completion of electrophoresis the gel was removed and stained with colloidal blue G (Sigma-Aldrich), a Coomassie blue based protein analysis stain, according to the manufacturer's instructions (Appendix 5). The gel was visualised and digitally photographed using a white light source that is incorporated in the gel image analyser Gel Doc 2000 system (Bio-Rad, UK) and analysed with Quantity One® software (Bio-Rad).

Precision Plus Protein standards (Bio-Rad) are precise recombinant prestained and unstained protein standards for electrophoresis and Western blotting. They are designed as high accuracy protein reference bands for determining molecular weights of unknown proteins. The molecular weight marker can resolve 10 bands corresponding to precise weight recombinant protein standards, but with the concentration of gel being used this was reduced to 9 bands. Markers were electrophoresed at 180V for 55 min on a 12 % separating acrylamide gel with Colloidal blue G staining. The 10 kDa marker is not represented on the gel as the time required for sample electrophoretic separation consistently ran the marker off the gel. Other molecular weight markers were used in the beginning of the experiments and were: Kaleidoscope polypeptide standards 3.7 – 34.9 kDa (Bio-Rad) and High and Low Range Polypeptide SDS-PAGE standards 14.3 – 200kDa (Bio-Rad).

PEOs and their constituents' effects on G. duodenalis cell biology

A concentration of 5 x 10^7 trophozoites ml^{-1} (equivalent to 3.75 x 10^6 trophozoites $lane^{-1}$) was found to provide the best protein resolution and was therefore used in the following experiments. The parasites were prepared as previously described for PEO screening and titrations in 96 well microtitre plates with oils of myrtle, elemi oils and their constituents (α-pinene, eucalyptol and limonene respectively) effects on protein

expression assessed after 24 h incubation (37°C) with viability determination using Trypan blue inclusion/exclusion. Controls included heat killed (56°C, 10 min), freeze killed (liquid nitrogen, 2 min) and metronidazole treated trophozoites. All samples were prepared in quintuplicate and the experiments performed in duplicate. Lysates for each sample were prepared as previously described. The prepared lysate was then either used immediately or frozen at -80°C for SDS-PAGE analysis.

Statistical analysis

In this series of experiments *Giardia* trophozoites from each microtitre well from 2 experiments, were counted giving a total of either 6 or 10 enumerations (where stated) for each PEO concentration tested. The means and standard deviations of these counts were used in formulating graphs and tables. A Student's 2-tailed, unpaired, t-test was used to determine significance with a *P* value of less than or equal to 0.05 being regarded as significant. The construction of dose–response curves and calculation of IC_{50} and IC_{90} values and statistical analysis was performed using XLfit 4® software and Microsoft Excel® software.

2:4 RESULTS

Ethanol and DMSO titrations

Incubation of *G. duodenalis* trophozoites in the presence of serial dilutions of DMSO at concentrations to be found with the diluted PEOs, significantly reduced their viability over a 24 h period (Table 2.2). Ethanol treatment, however, did not affect trophozoites viability (Table 2.2). Viable (motile and sessile) trophozoites excluded Trypan blue. Motile trophozoites exhibited forward movement during which they rotated around their longitudinal axis displaying both a tumbling movement resembling that of a falling leaf and an up and down movement known as 'skipping'. Sessile (viable) trophozoites excluded Trypan blue and retained their typical pyriform shape and internal morphology (Figure 2.1, panels 1 - 3). Dead trophozoites included Trypan blue and often their typical pyriform shape became either slightly misshapen or rounded and swollen (Figure 2.1, panels 4 - 9).

DMSO when used at concentrations equivalent to that found in a 0.02% dilution of PEO significantly reduced the viability of trophozoites in a dose dependent manner (Table 2.2, $P < 0.05$). Ethanol, however, was not found to have any significant effect on trophozoite viability or morphology and was used as the diluent for PEOs in experiments (Table 2.2, $P > 0.05$).

Incubation of G. duodenalis trophozoites with metronidazole

Incubation of *G. duodenalis* trophozoites in the presence of serial dilutions of the reference drug, metronidazole, significantly reduced their viability over a 24 h period. A standard dose response curve was observed and the minimum concentration of metronidazole required to kill 100% of trophozoite *in vitro* was determined to be 67 μg ml^{-1} (Figure 2.2). This concentration of reference drug was then used a standard by which PEOs, soft fruit extracts and their constituents could be compared for all experiments using *Giardia* trophozoites.

Table 2.2 *In vitro* viability of *G. duodenalis* trophozoites using varied concentrations of ethanol and DMSO.

	Viability of Trophozoites (%)	
Concentration (% v/v)	**Ethanol**	**DMSO**
0	4.49 ± 2.0	4.49 ± 2.0
0.0067	6.38 ± 2.5	19.35 ± 16.6
0.0167	5.23 ± 2.6	25.09 ± 21.3
0.033	4.20 ± 2.3	39.64 ± 31.0
0.067	8.62 ± 5.6	60.72 ± 36.5

Trophozoites were incubated for 24 h in the presence of either ethanol or DMSO (0, 0.067, 0.033, 0.0167 and 0.0067%) and their viability assessed.

Incubation of G. duodenalis trophozoites with PEOs

Screening of PEOs

Incubation of *G. duodenalis* trophozoites in the presence of PEOs at a final concentration of 0.02% v/v or metronidazole at 67 µg ml^{-1} were equally effective at reducing trophozoite viability by 100% as assessed by TBMM (Appendix 6).

Dead trophozoites all had similar morphology, retaining a pyriform shape or were rounded and swollen or had evidence of blebbing. In metronidazole treated *G. duodenalis* trophozoites, the nuclei were enlarged and darkly stained, cytoplasmic staining also appeared 'grainy' possibly due to vacuole formation with the trophozoite which retained its pyriform morphology. Blebbing was also evident (Figure 2.3 A). Representative examples of trophozoite morphology caused by PEOs are shown in panels B – E. All panels show a 'grainy' cytoplasm with nuclei being slightly enlarged and darkly stained. Treatment of trophozoites with patchouli oil affected their morphology, causing the swelling and rounding up of trophozoites (Figure 2.3 B). Myrrh treated trophozoites retained their pyriform shape and also showed evidence of blebbing (Figure 2.3 C), as did those treated with elemi (Figure 2.3 E). The greatest change to trophozoite morphology was caused by myrtle (Figure 2.3 D) with all trophozoites becoming swollen, rounded, blebbed and with large vacuole formation occurring in the cytoplasm.

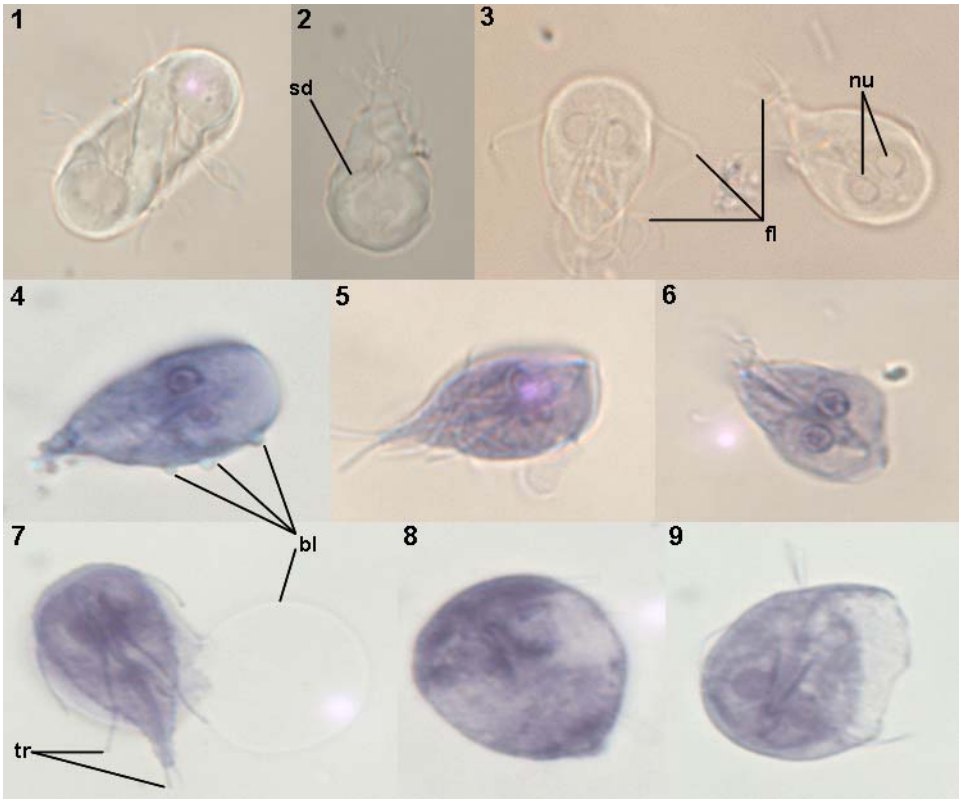


Figure 2.1 *In vitro* viability of *G. duodenalis* trophozoites as assessed by TBMM. Trophozoites were incubated for 24 h in the presence or absence of ethanol or DMSO (0, 0.067, 0.033, 0.0167 and 0.0067%) and viable trophozoites were viewed under bright field microscopy (x1250 total magnification). Viable *G. duodenalis* trophozoites are shown in division (panel 1); in ventral view, showing typical pyriform appearance and ventral sucking disc (**sd**) (panel 2); in dorsal view, exhibiting flagella (**fl**) and the 2 characteristic nuclei (**nu**) (panel 3). Dead trophozoites included Trypan blue, often retaining their pyriform shape. Some were slightly misshapen (panels 4 -7) or swollen (panels 8 and 9). In some trophozoites, blebbing (**bl**) was evident (panels 4 and 7) and flagella appeared truncated (**tr**) (panel 7). DMSO treatment caused changes in trophozoite morphology and panels 4 – 9 show typical examples of these changes. Photographs were taken (copyright© SPDL) using a ColorView Soft Imaging System MTV-3 camera and analySIS® software.

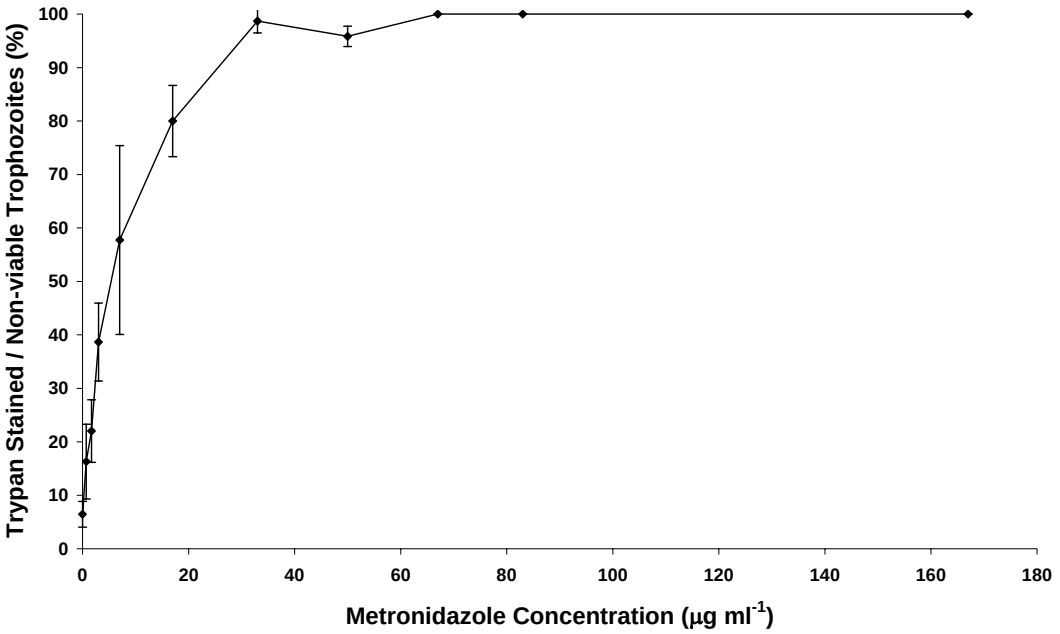


Figure 2.2 *In vitro* viability of *G. duodenalis* trophozoites using varied concentrations of metronidazole.
Trophozoites were incubated for 24 h in the presence of different concentrations of the reference drug, metronidazole (0 – 167 μg ml^{-1}) and their viability assessed by TBMM.

Titration of PEOs

As all PEOs were found to be equally effective at killing the trophozoites at 0.02%, titrations were performed to determine the MIC of PEOs required to kill 100% of trophozoites *in vitro*. Initial titrations were carried out from 0.02% to 0.005% in doubling dilutions where it was discovered that only a few PEOs retained a 100% killing level at the lowest concentration of 0.005% (Figure 2.4). These PEOs were myrrh, myrtle and elemi. Oils of marjoram, palmarosa, thyme and geranium all had an MIC of 0.01% with sweet fennel, lavender, amyris, patchouli and buchu oil having an MIC of 0.02% (Figure 2.4 and Table 2.3).

As myrrh, myrtle and elemi still elicited a 100% killing effect on *G. duodenalis* trophozoites, a further titration of doubling dilutions was performed with these PEOs to determine their MIC. Plant oil activity was found to be dose dependant with all three oils failing to completely reduce trophozoite viability at concentrations lower than 0.005% (Figure 2.5). From this it was deduced that the MIC for myrrh, myrtle

and elemi was 0.005% although > 80% of trophozoites were non-viable with 0.0025% myrrh which then dropped to < 40% with 0.00125% myrrh. The change from trophozoites being viable to non-viable was more marked with oils of myrtle and elemi where at 0.0025% both oils caused ~30% trophozoite death, a 70% increase in viability from 0.005% (Figure 2.5).

The MIC for myrrh, myrtle and elemi oils (0.005%) compares favourably with that of metronidazole (67 μg ml^{-1}) when one converts the percentage concentration values for PEOs into μg ml^{-1}. If 1 μg ml^{-1} is equal to 1 part per million then 1 ppm is equal to 1/1,000,000 which is equal to 0.0001%. Since the MIC for the 3 most effective oils was 0.005% this is equal to 50 μg ml^{-1}. This is actually lower than that calculated for metronidazole and would indicate that these oils at a concentration of 0.005% (or 50 μg ml^{-1}) are more effective than metronidazole. However, metronidazole has a greater effect on trophozoite viability at lower concentrations than any of the oils tested where the 90% and 50% inhibitory concentrations (IC_{90} and IC_{50}) were calculated to be 21.8 μg ml^{-1} and 5.6 μg ml^{-1} respectively (Table 2.3). All the PEOs tested failed to reach these levels and, collectively, had a wide variety of activity (IC_{90} = 0.003 – 0.013 % [30 – 130 μg ml^{-1}], IC_{50} = 0.0009 – 0.007 % [9 – 70 μg ml^{-1}], Table 2.3). The most effective oil at the IC_{90} and IC_{50} level was not myrrh, myrtle or elemi but actually palmarosa oil (IC_{90} = 0.003 % [30 μg ml^{-1}], IC_{50} = 0.0009 % [9 μg ml^{-1}], Table 2.3). This would indicate that palmarosa has a very narrow range of activity when compared to myrrh, myrtle or elemi on an individual basis.

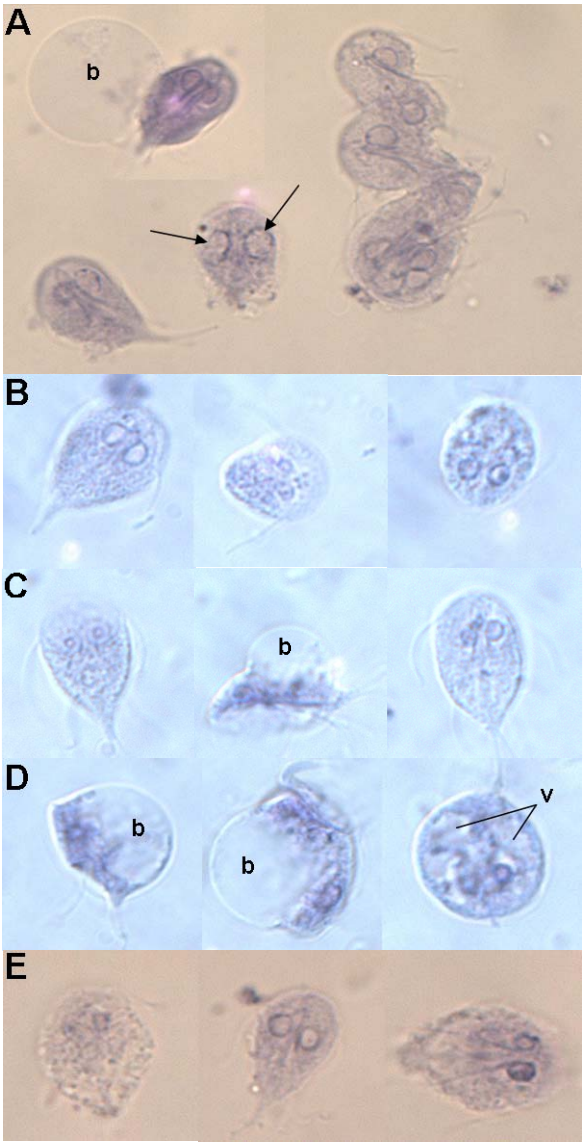


Figure 2.3 *In vitro* viability of *G. duodenalis* trophozoites incubated with PEOs for light microscopy.
Trophozoites were incubated for 24 h in the presence or absence of a final concentration of 0.02% PEO or 67 μg ml^{-1} of metronidazole and their viability assessed by TBMM. Panel **A** shows the effects of metronidazole on *G. duodenalis* trophozoites. The nuclei are enlarged (**arrows**) and darkly stained, cytoplasmic staining also appears 'grainy' possibly due to vacuole formation with the trophozoite retaining it pyriform morphology. Blebbing was also evident (**b**). Representative examples of trophozoite morphology induced by PEOs are shown in panels **B** – **E**. All panels show a 'grainy' cytoplasm with nuclei being slightly enlarged and darkly stained with patchouli treated parasites being pyriform to rounded and swollen (**B**). Both myrrh (**C**) and elemi (**E**) treated trophozoites maintained a pyriform shape, but with blebbing. Large vacuole formation occurred in the cytoplasm (**v**) of myrtle treated trophozoites (**D**). Photographs were taken (copyright© SPDL) using a ColorView Soft Imaging System MTV-3 camera and analySIS® software.

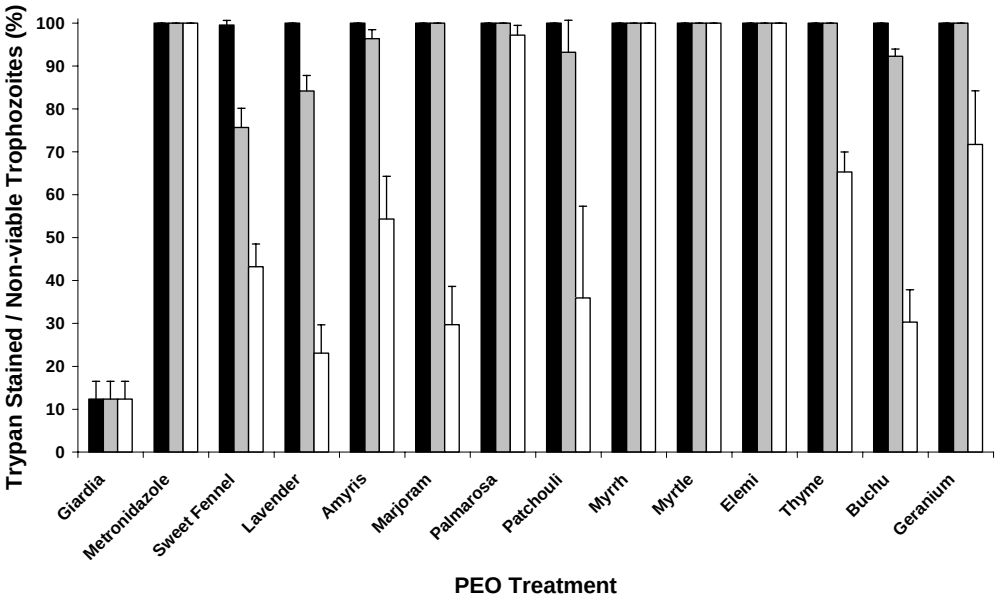


Figure 2.4 *In vitro* viability of *G. duodenalis* trophozoites incubated with a titration of PEOs.
Trophozoites were incubated for 24 h in the presence or absence of a final concentration of 0.02% (black bars), 0.01% (grey bars), 0.005% (white bars) PEO or 67 μg ml^{-1} metronidazole and their viability assessed by TBMM.

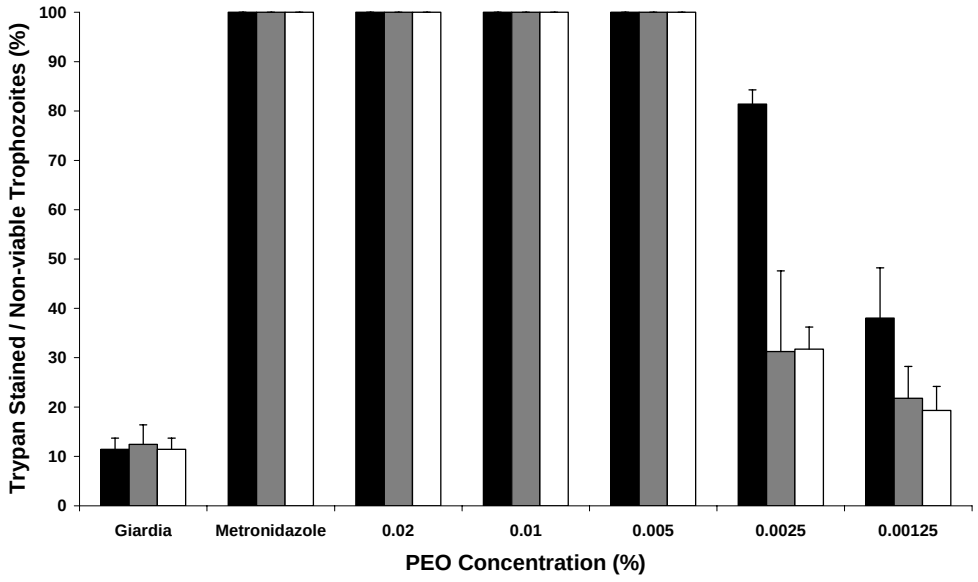


Figure 2.5 *In vitro* viability of *G. duodenalis* trophozoites incubated with myrrh, myrtle or elemi oils.
Trophozoites were incubated for 24 h in the presence or absence of myrrh (black bars), myrtle (grey bars), elemi (white bars) PEO or 67 μg ml^{-1} of metronidazole and their viability assessed by TBMM. The PEOs were titrated in doubling dilutions from 0.02% - 0.00125% final concentrations.

Table 2.3 Inhibition of *G. duodenalis* trophozoite survival by different PEOs

	INHIBITORY CONCENTRATION OF PEO					
PEO	**MIC[a]**	**MIC[b]**	**IC_{90}[a]**	**IC_{90}[b]**	**IC_{50}[a]**	**IC_{50}[b]**
Metronidazole	67 μg ml^{-1}	67 μg ml^{-1}	21.8 μg ml^{-1}	21.8 μg ml^{-1}	5.6 μg ml^{-1}	5.6 μg ml^{-1}
Amyris	0.02	200	0.009	90	0.004	40
Buchu	0.02	200	0.01	100	0.006	60
Lavender	0.02	200	0.011	110	0.007	70
Patchouli	0.02	200	0.0097	97	0.006	60
Sweet Fennel	0.02	200	0.013	130	0.006	60
Geranium	0.01	100	0.007	70	0.003	30
Marjoram	0.01	100	0.009	90	0.006	60
Palmarosa	0.01	100	0.003	30	0.0009	9
Thyme	0.01	100	0.0075	75	0.004	40
Elemi	0.005	50	0.0048	48	0.003	30
Myrtle	0.005	50	0.0049	49	0.003	30
Myrrh	0.005	50	0.003	30	0.0015	15

[a] Concentrations are given as a percentage v/v.
[b] Concentrations are given as μg ml^{-1} where 0.0001% = 1 ppm = 1 μg ml^{-1}.
MIC = the minimum concentration required to inhibit the growth/culture of 100% trophozoites.
IC_{90} and IC_{50} = the concentration required to inhibit the growth/culture of 90% or 50% of trophozoites respectively.
Viability was assessed using TBMM.

Incubation of G. duodenalis trophozoites with PEO constituents

The 3 PEOs having the lowest MIC were examined to determine their chemical composition with the assistance of F.D. Copeland and Sons. This company used gas chromatography coupled with mass spectroscopy to elucidate chemical compositions for the oils of myrrh, myrtle and elemi (Appendix 3). Myrrh had a complex composition with over 50 individual constituents being found. Unfortunately the constituents in greatest abundance (the furanic sesquiterpenes furanodiene [31.97%] and curezene [26.69%]) were unavailable for purchase from chemical manufacturers. This meant that no further investigations into the activity of these constituents could be performed.

In the case of myrtle oil the major constituents were found to be the monoterpenes α-pinene and eucalyptol (syn. 1,8-cineole) comprising 51.72% and 22.35% of the whole oil respectively. The main constituents of elemi oil composition were found to be the terpene, limonene (51.54%; syn. (+)-*p*-Mentha-1,8-diene; (+)-Carvene; (*R*)-4-Isopropenyl-1-methyl-1-cyclohexene), α-phellandrene (16.23%) and elemol

(10.90%). Whilst both the main constituents of myrtle were available for purchase only limonene could be obtained for elemi oil.

Oil constituents were used at concentrations equivalent to that found in the whole oil such that if the constituent comprised 51.72% of the oil then the constituent, when tested on its own, would be used at a concentration 51.72% of the whole oil concentrations, i.e. 0.0026%.

When the constituents were initially used at the equivalent screening concentration of the oils at 0.02% both α-pinene and limonene reduced the viability of *G. duodenalis* trophozoites by 100% (Figure 2.6). The second constituent of myrtle oil was 1,8-cineole and when tested at a concentration of 0.0045% (its equivalent concentration found in 0.02% of the whole oil) reduced trophozoite viability by only 17%. This would indicate that the active constituent responsible for the death of *G. duodenalis* trophozoites *in vitro* in myrtle oil was α-pinene and not eucalyptol. Maximum killing was still observed when both these constituents were used in combination suggesting that eucalyptol did not have any agonistic or antagonistic effects upon the action of α-pinene. Limonene was the only one constituent of elemi available for testing and when used at a final concentration of 0.01% killed 100% of *G. duodenalis* trophozoites. Limonene would appear to be the active constituent found in elemi oil.

However, when the constituents were examined at the MIC levels found in their respective whole oils, i.e. 0.005%, all constituents failed to significantly reduce the viability of *G. duodenalis* trophozoites when compared with untreated controls (Figure 2.7).

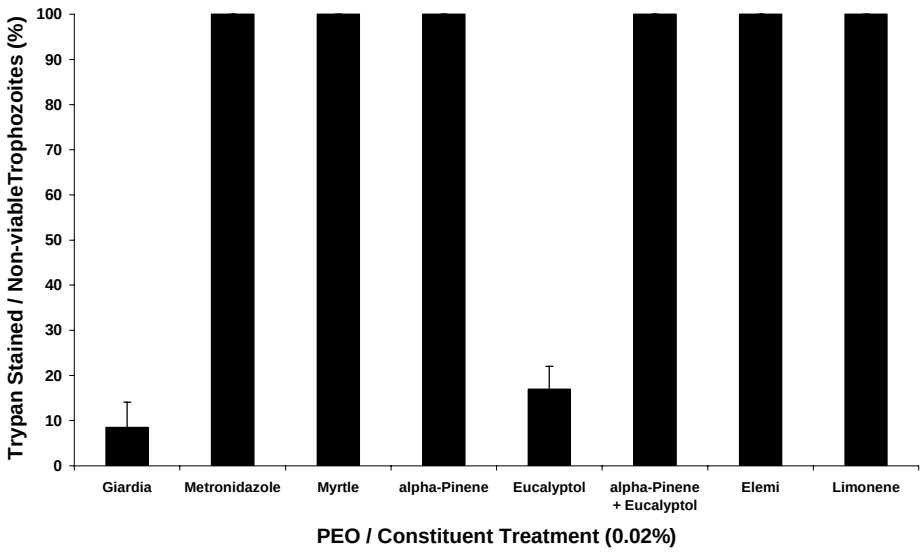


Figure 2.6 *In vitro* viability of *G. duodenalis* trophozoites incubated with myrtle or elemi oils and their constituents at screening concentrations.
Trophozoites were incubated for 24 h in the presence or absence of 0.02% myrtle, 0.02% elemi PEO, 0.01% α-pinene, 0.0045% eucalyptol, a combination of α-pinene and eucalyptol, 0.01% limonene or 67 µg ml^{-1} of metronidazole and their viability assessed by TBMM.

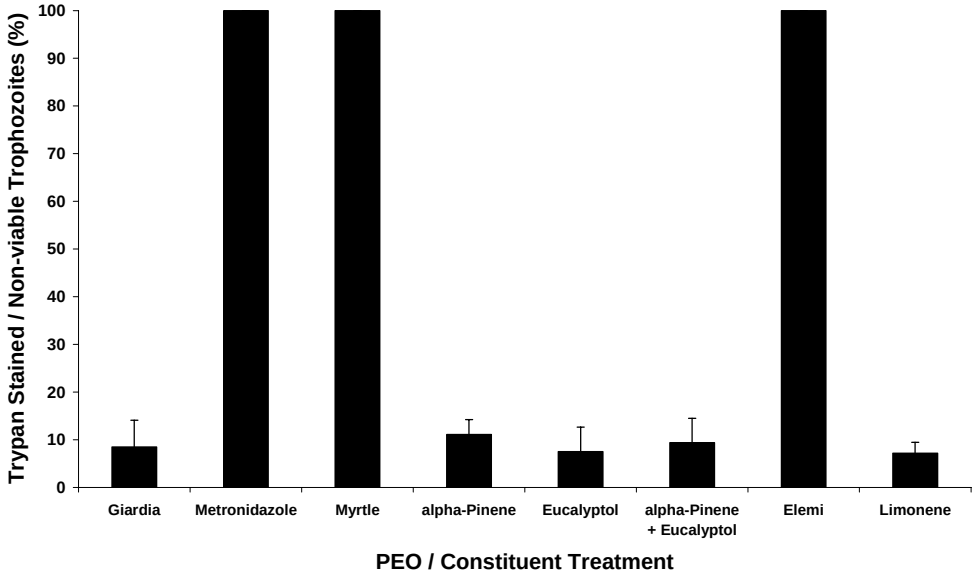


Figure 2.7 *In vitro* viability of *G. duodenalis* trophozoites incubated with myrtle or elemi oils and their constituents at MIC concentrations.
Trophozoites were incubated for 24 h in the presence or absence of 0.005% myrtle, 0.005% elemi PEO, 0.0026% α-pinene, 0.0011% eucalyptol, a combination of α-pinene and eucalyptol, 0.0026% limonene or 67 µg ml^{-1} of metronidazole and their viability assessed by TBMM.

Incubation of G. duodenalis trophozoites with PEOs and their constituents over a 2 h time course

It was noted that some parasiticidal action occurred within 120 min of incubation when a visual inspection of the microtitre plates being incubated for 24 h was routinely performed to determine adherence and general viability of trophozoites before being left to continue incubation. Whilst this was anecdotal evidence of trophozoites starting to round up, losing motility and swelling, an experiment was designed to quantify this activity. The MICs of each PEO and selected constituents were used and the viability of trophozoites was assessed after 30, 60 and 120 min incubation.

All PEOs with the exception of myrrh killed 100% of trophozoites within 60 min of incubation at their MICs (Figure 2.8). This was significantly different from metronidazole which failed to kill 100% of trophozoites when compared to the untreated controls at all three incubation times tested (Figure 2.8, $P > 0.05$). Metronidazole did, however, reduce trophozoite viability after 24 h (Figure 2.9). Oils of lavender (MIC = 0.02%), marjoram (MIC = 0.01%), palmarosa (MIC = 0.01%) and geranium (MIC = 0.01%) were able to kill 100% of trophozoites after only 30 min (Figure 2.8). The difference in the activity between the oils may be due to differences in their constituents.

There was no significant difference in the activities of α-pinene, eucalyptol (myrtle constituents) and limonene (elemi constituent) on trophozoite viability throughout the incubation times tested (30 min to 24 h). All failed to significantly change trophozoite viability from untreated control levels (Figure 2.9).

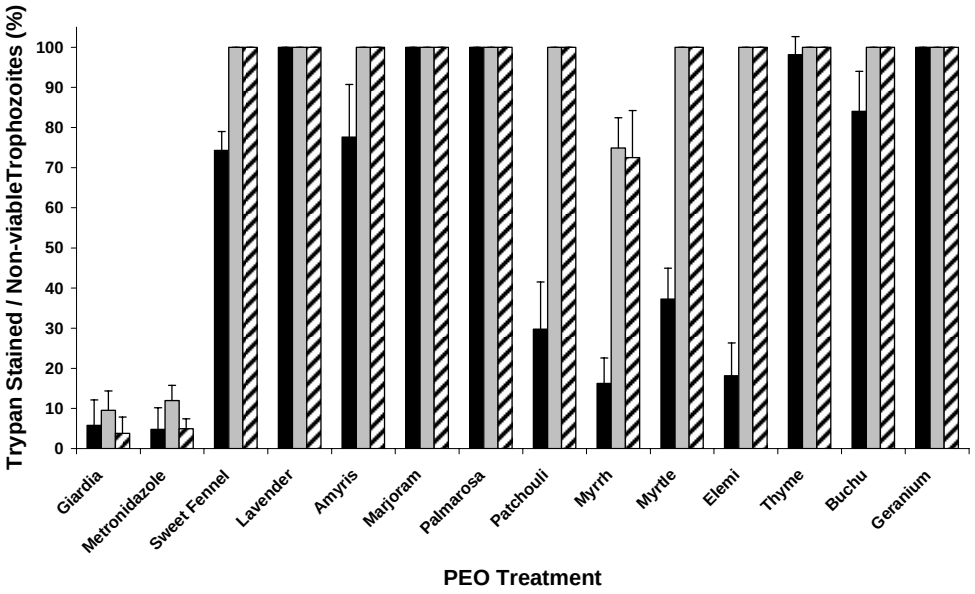


Figure 2.8 *In vitro* viability of *G. duodenalis* trophozoites incubated with PEOs over a 2 h time course.
Trophozoites were incubated for up to 2 h in the presence or absence of PEO at a final concentration equivalent to the PEO pre-determined MIC. Viability of trophozoites was assessed after 30 min incubation (black bars), 60 min (grey bars), 120 min (white bars) of PEO or 67 μg ml^{-1} metronidazole and their viability assessed by TBMM.

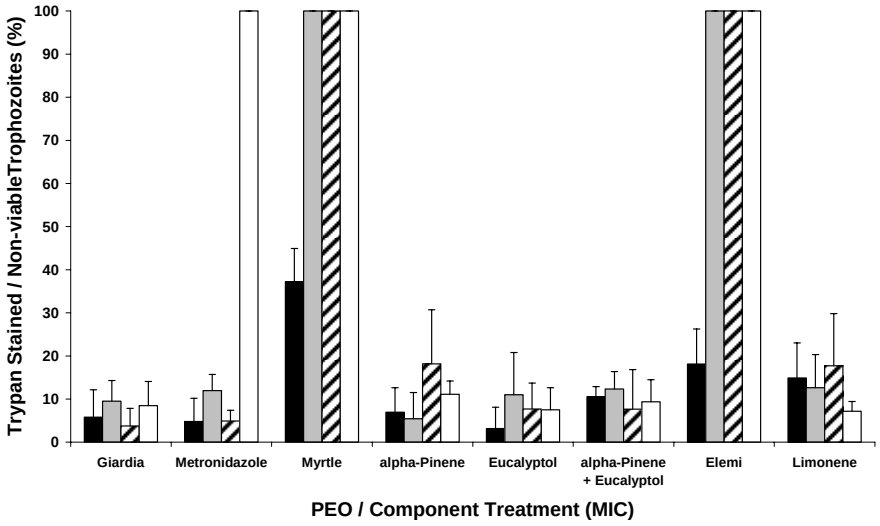


Figure 2.9 *In vitro* viability of *G. duodenalis* trophozoites incubated with PEOs and constituents over a 2 h time course.
Trophozoites were incubated for up to 24 h in the presence or absence of myrtle, elemi or their main constituents at a final concentration equivalent to the oils pre-determined MIC. Viability of trophozoites were assessed after 30 min incubation (black bars), 60 min (grey bars), 120 min (diagonal bars) or 24 h (white bars) PEO or 67 μg ml^{-1} metronidazole and their viability assessed by TBMM.

Trophozoite protein profiling by SDS-PAGE analysis

Lysate titration – SDS PAGE analysis

In order to be able to visualise a protein profile from a parasite lysate, one requires a minimum amount of protein which can be stained and analysed. A titration of *G. duodenalis* trophozoite lysate concentrations for use in SDS-PAGE analysis was performed. Increasing the concentration of parasites increased the amount of protein available for staining in the SDS-PAGE gel. Concentrations of parasites ranged from 2.7×10^5 ml^{-1} up to 5×10^7 ml^{-1}. No protein profiles were visible with Colloidal blue G staining until at least 1×10^7 ml^{-1} trophozoites were used to prepare lysates and optimal visualisation occurred with 5×10^7 ml^{-1}(data not shown). This concentration was then used in all SDS-PAGE analysis experiments. This was the equivalent of 3.75×10^6 trophozoites $lane^{-1}$.

Heat and freeze killing of trophozoites

Viability assessment

A positive control for killing trophozoites was required. Whilst metronidazole was used in previous experiments, controls that produced a protein profile similar to that of untreated trophozoites were sought. Two methods for the rapid killing of trophozoites were used, heat killing and freeze killing, in order to make comparisons with metronidazole and PEO effects on trophozoite protein profile.

Live trophozoites were subjected to heat treatment (56°C for 10 min) or freeze treatment (liquid nitrogen, 2 min) and their viability assessed by TBMM. In both treatment regimens all trophozoites included Trypan blue indicating non-viability with a swollen morphology and were non-motile. Trophozoite plasma membrane permeability was increased as indicated by the uptake of Trypan blue, but no other disruption to the trophozoite plasma membrane was observed, with the parasites remaining intact (Figure 2.10).

SDS-PAGE analysis

Once viability had been assessed, lysates were prepared from the trophozoites, fractionated by SDS-PAGE and stained with Colloidal blue G. Heat killing (HK)

trophozoites caused changes to the protein profile when compared to untreated trophozoite controls (Figure 2.11). Some proteins had greater expression with HK compared to untreated trophozoites as indicated by more intensely stained bands (Figure 2.11). In both HK and FK treatments a band corresponding to ~100 kDa was upregulated compared to the untreated control. A 37 kDa protein was absent when trophozoites were freeze killed but was present in untreated trophozoites and upregulated in heat killed trophozoites indicating that this protein is possibly a heat shock protein or closely related to that class of proteins due to its upregulation in heat treated trophozoites. Untreated trophozoites showed molecular masses ranging from ~25 – 250 kDa.

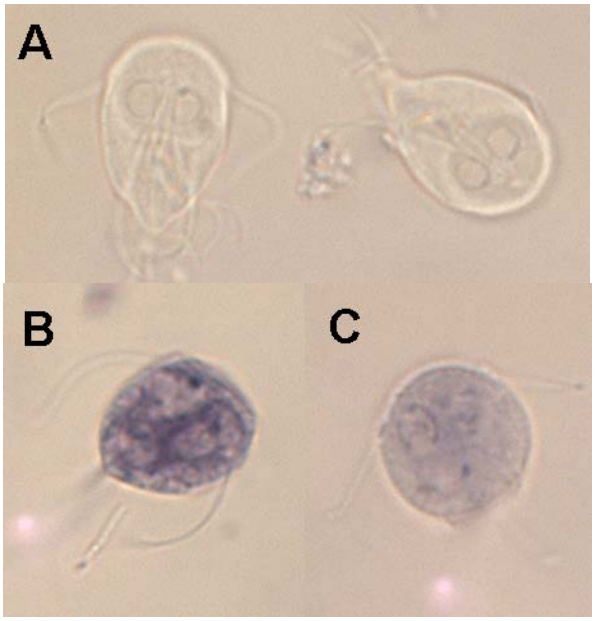


Figure 2.10 The effect of heat and freeze killing on *G. duodenalis* trophozoites. Trophozoites were subjected to heat treatment (**HK**; 56°C, 10 min) or freezing (**FK**; liquid nitrogen, 2 min) and their viability assessed by TBMM. Representative photographs are shown for each method. Untreated trophozoites had typical pyriform morphology and excluded Trypan blue stain (**A**). Both heat killed trophozoites (**B**) and freeze killed trophozoites (**C**) showed an uptake of Trypan blue and had rounded morphology. Photographs were taken (copyright© SPDL) using a ColorView Soft Imaging System MTV-3 camera and analySIS® software.

Metronidazole titration – SDS PAGE analysis

Incubation of trophozoites with increasing concentrations of metronidazole did not change the outcome of viability. The reference drug was just as effective in killing trophozoites at the final concentration of 20 µg with the high numbers of parasites

used in this protein analysis experiments in comparison to the previous viability studies (5 x 10^7 ml^{-1} compared to 2.7 x 10^5 ml^{-1}). SDS-PAGE analysis shows no difference in the protein profile at each concentration of metronidazole used (Figure 2.12). However, metronidazole appears to enhance the production of a ~100kDa protein when compared with untreated controls. This was also observed for heat and freeze killed trophozoites indicating that this protein is upregulated during death by a variety of means.

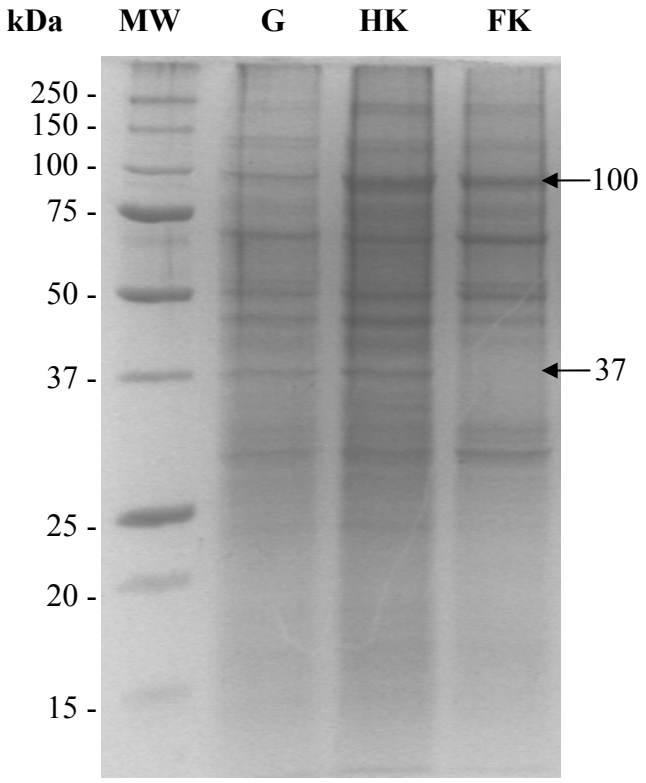


Figure 2.11 The effect of heat and freeze killing on *G. duodenalis* trophozoite protein profile using SDS-PAGE analysis.
Trophozoites were subjected to heat treatment (**HK**; 56°C, 10 min) or freezing (**FK**; liquid nitrogen, 2 min) and lysates were prepared. Lysates were compared with untreated trophozoites (**G**) and were loaded on a 12% acrylamide separating gel and 4% acrylamide stacking gel and electrophoresed at 180V for 55 min. Protein bands are visualised using the Colloidal blue G staining system and run against a molecular weight marker (**MW**; Bio-Rad).

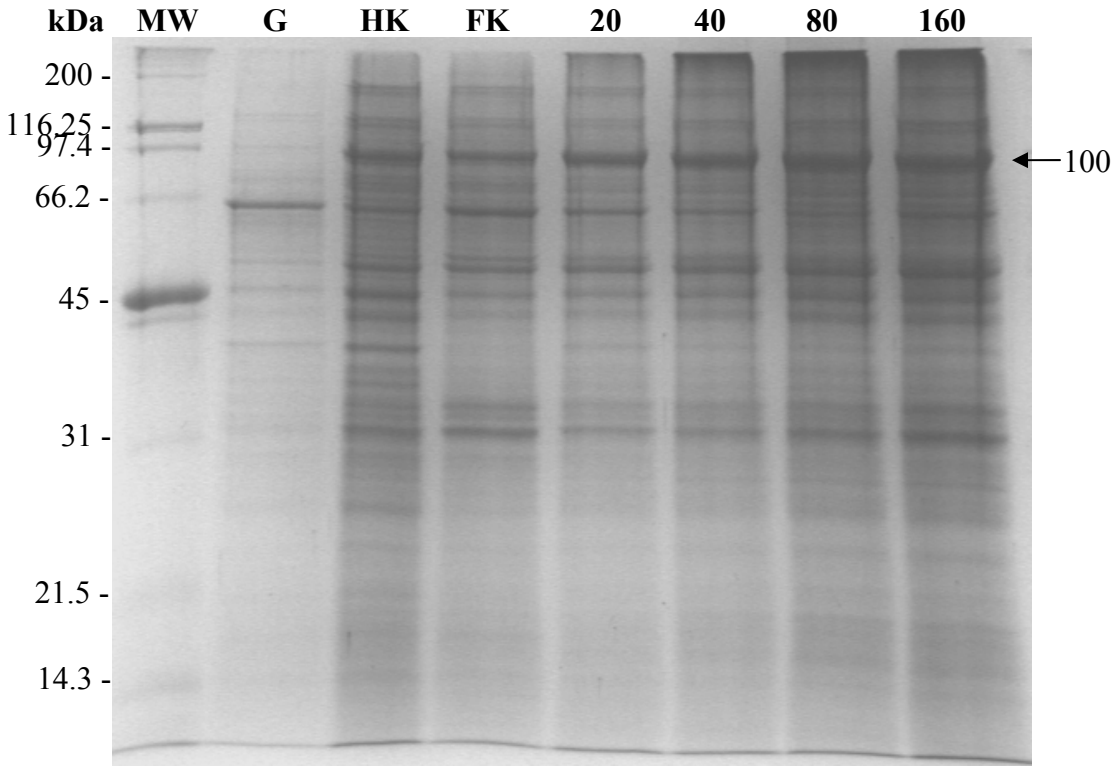


Figure 2.12 Titration of metronidazole for use in SDS-PAGE analysis.
G. duodenalis trophozoites (5 x 10^7 ml^{-1}) were incubated in the presence or absence of final concentrations 0, 20, 40, 80 or 160 µg of metronidazole in 96 well microtitre plates and compared with untreated trophozoites (**G**). Heat killed (**HK**) and freeze killed (**FK**) trophozoite lysates were prepared as other controls for trophozoite killing. All lysates prepared were loaded on a 12% acrylamide separating gel and 4% acrylamide stacking gel and electrophoresed at 180V for 55 min. Protein bands are visualised using the Colloidal blue G staining system and run against a molecular weight marker (**MW**; High and Low weight range polypeptide markers Bio-Rad).

PEOs and their constituents' effect on G. duodenalis cell biology

Effect of myrtle oil on viability as assessed by TBMM and SDS PAGE analysis

Incubation of 5 x 10^7 ml^{-1} trophozoites in the presence of myrtle oil at concentrations used in previous experiments (0.02, 0.01, 0.005 and 0.0025% final concentrations) elicited similar responses in that there was a concentration dependant reduction in trophozoite viability (Table 2.4). Of note is that using this higher concentration of trophozoites (5 x 10^7 ml^{-1} instead of 2.67 x 10^5 ml^{-1}) than used in previous PEO experiments, the MIC was changed from 0.005% to 0.01% for the previous PEO experiments. In Table 2.4 it can be seen that there is a small, but significant (2.4 ± 2.2%; $P < 0.05$) percentage of trophozoites still viable at the previously determined

MIC of 0.005%. This phenomenon was also observed with elemi oil treated trophozoites under the same conditions (Table 2.4).

SDS-PAGE analysis of these myrtle treated trophozoites show a concentration dependant increase in the upregulation of proteins of the following molecular weights: 200, 150, 100, 50 and ~30 kDa (Figure 2.13). Killing of trophozoites with myrtle oil caused the specific upregulation of a 200 and a 150 kDa protein. All other killing treatments (heat, freezing and chemotherapeutic) did not affect these proteins and as the concentration of myrtle oil was reduced, so was the production of this protein as seen by a reduction in the intensity of the protein band staining. This was also evident with the 50 and the ~30 kDa protein. Treatment of trophozoites with myrtle oil did not just cause the upregulation of proteins but also caused their abrogation. Both the 37 kDa and a ~70 kDa protein were inhibited by high concentrations of myrtle oil (0.02 and 0.01%) and started to reappear at the lower concentrations used (0.005 and 0.0025%). This is in accordance with the Trypan blue data which suggested that there were some viable trophozoites present when treated with 0.005% and 0.0025% myrtle oil. The SDS-PAGE analysis details a reappearance of these proteins (37 and ~70 kDa) as the number of viable trophozoites increase with a decrease in myrtle oil concentration. Only myrtle oil abolishes the ~70 kDa protein, with this protein found at equal intensities in untreated trophozoites and in heat, freeze and metronidazole killed trophozoites. The 37 kDa protein abolished by myrtle oil was also abolished by the actions of freeze killing and metronidazole treating the trophozoites.

Table 2.4 A comparison between the effect of myrtle and elemi oil on *G. duodenalis* trophozoite viability using different concentrations of parasites

PEO Concentration (%)	Inhibition of trophozoites (%)			
	Parasite Concentration (5×10^7 ml^{-1})		Parasite Concentration (2.67×10^5 ml^{-1})	
	Myrtle	Elemi	Myrtle	Elemi
0.02	100 ± 0	100 ± 0	100 ± 0	100 ± 0
0.01	100 ± 0	100 ± 0	100 ± 0	100 ± 0
0.005	97.6 ± 2.2	96.9 ± 3.4	100 ± 0	100 ± 0
0.0025	69.4 ± 9.3	75.8 ± 7.2	31.3 ± 4.5	31.8 ± 4.5

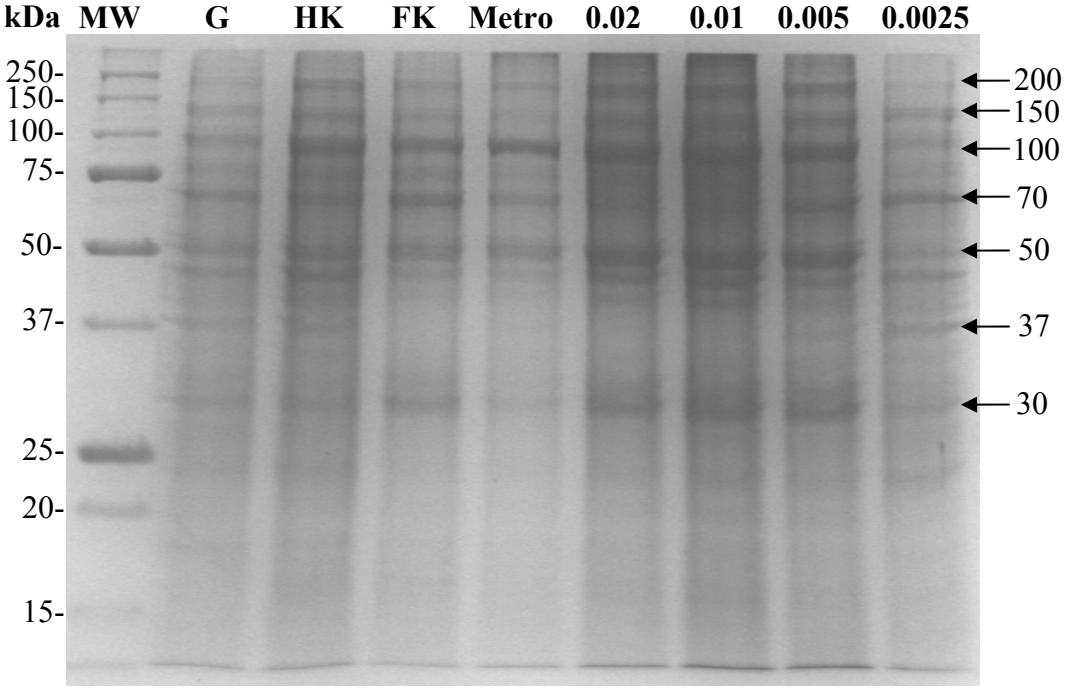


Figure 2.13 The effect of myrtle oil on *G. duodenalis* trophozoite protein profile using SDS-PAGE analysis.
G. duodenalis trophozoites (**G**; 5×10^7 ml^{-1}) were incubated in the presence or absence of final concentrations of 20 µg metronidazole (**Metro**.) and 0.0025 – 0.02% myrtle oil in 96 well microtitre plates and compared with untreated trophozoites (**G**). Heat killed (**HK**) and freeze killed (**FK**) trophozoite lysates were prepared as other controls for trophozoite killing. All lysates prepared were loaded on a 12% acrylamide separating gel and 4% acrylamide stacking gel and electrophoresed at 180V for 55 min. Protein bands are visualised using the Colloidal blue G staining system and run against a molecular weight marker (**MW**; Bio-Rad).

Effect of elemi oil on viability as assessed by TBMM and SDS PAGE analysis

As observed with the myrtle oil treated trophozoites incubation of 5 x 10^7 ml^{-1} trophozoites in the presence of elemi oil at concentrations used in previous experiments elicited similar responses in that there was a concentration dependant reduction in trophozoite viability (Table 2.4). As seen previously with myrtle oil, using a higher concentration of trophozoites (5 x 10^7 ml^{-1} instead of 2.67 x 10^5 ml^{-1}) than used in previous PEO experiments changes elemi oils MIC from 0.005% to 0.01%. In Table 2.4 it can be seen that there is a small but significant percentage (3.1 ± 3.4%; $P < 0.05$) of trophozoites still viable at the previously determined MIC of 0.005%.

SDS-PAGE analysis of these elemi treated trophozoites shows a concentration dependant increase in band intensities of proteins of the following molecular weights: 200, 150, 50 and ~30 kDa (Figure 2.14).

Killing of trophozoites with elemi oil caused the specific upregulation of a 200 and a ~30 kDa protein (Figure 2.14). All other killing treatments (heat, freezing and chemotherapeutic) did not affect these proteins and as the concentration of elemi oil was reduced, so was the production of this protein as seen by a reduction in the intensity of the protein band staining. This is in accordance with the Trypan blue data which suggested that there were some viable trophozoites present when incubated with 0.005% and 0.0025% myrtle oil. The SDS-PAGE analysis details an increase of these proteins as the percentage of viable trophozoites increase with a concurrent decrease in myrtle oil concentration. Treatment of trophozoites with elemi oil did not only cause the upregulation of proteins, it also caused their inhibition. The 37 kDa protein was inhibited with all concentrations of elemi oil. The 100 kDa protein was upregulated at all concentrations of elemi oil used. This was also shown in heat, freeze and metronidazole killed trophozoites. Untreated trophozoites expressed this protein less.

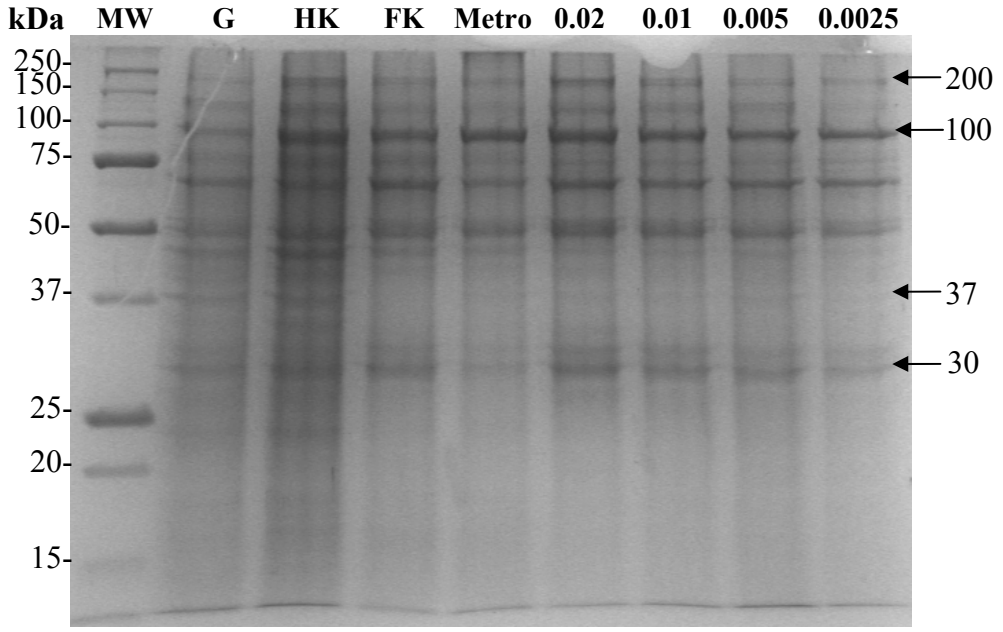


Figure 2.14 The effect of elemi oil on *G. duodenalis* trophozoite protein profile using SDS-PAGE analysis.
G. duodenalis trophozoites (**G**; 5 x 10^7 ml^{-1}) were incubated in the presence or absence of final concentrations of 20 µg metronidazole (**Metro.**) and 0.0025 – 0.02% elemi oil in 96 well microtitre plates and compared with untreated trophozoites (**G**). Heat killed (**HK**) and freeze killed (**FK**) trophozoite lysates were prepared as other controls for trophozoite killing. All lysates prepared were loaded on a 12% acrylamide separating gel and 4% acrylamide stacking gel and electrophoresed at 180V for 55 mins. Protein bands are visualised using the Colloidal blue G staining system and run against a molecular weight marker (**MW**; Bio-Rad).

Effect of oil constituents on viability as assessed by TBMM and SDS PAGE analysis

As previous experiments determined that a concentration of 5 x 10^7 ml^{-1} trophozoites required a greater concentration of PEO to reach an MIC than with 2.7 x 10^5 ml^{-1} trophozoites, each constituent examined were used at a concentration equivalent to 0.02% of the whole oil.

The main constituents of myrtle oil, α-pinene and eucalyptol (determined in Appendix 3), both failed to completely inhibit trophozoite viability as determined by Trypan blue inclusion/exclusion. Whilst both these constituents failed to completely inhibit *G. duodenalis* when used individually, in combination the inhibitory effect was increased and although this increase was slight, it was significant when compared to the inhibitory effect of α-pinene and eucalyptol alone (Figure 2.15; $P <$

0.05). This effect though, was less than additive and not synergistic. The main constituent of elemi, limonene, also failed to completely inhibit trophozoite viability.

The most interesting result from SDS-PAGE analysis of these constituent treated trophozoites is that the inhibition of a 37 kDa protein seen in elemi and myrtle oil treated trophozoites was not observed when trophozoites were treated with their constituents, α-pinene, eucalyptol and limonene (Figures 2.16 and 2.17). This suggests that this protein is not inhibited by these constituents and that the effects of another constituent in the oil or a combination of all the constituents in the oil are responsible for this abrogation. In common with all treatments however, is the increase in expression of a 100 kDa protein suggesting that this is a protein that is possibly expressed during the death of trophozoites irrespective of the methods used to kill the trophozoites.

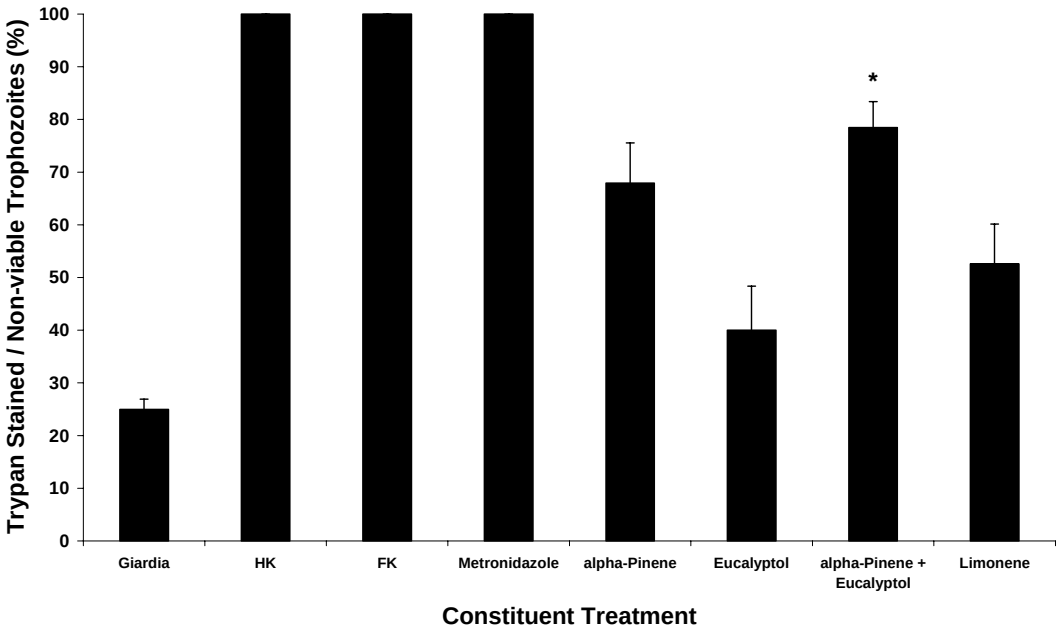


Figure 2.15 *In vitro* viability of *G. duodenalis* trophozoites incubated with the constituents of myrtle or elemi oil.
Trophozoites were incubated for 24 h in the presence or absence of 0.01% α-pinene, 0.0045% eucalyptol, a combination of α-pinene and eucalyptol, 0.01% limonene or 67 μg ml^{-1} of metronidazole and their viability assessed. Heat killed (HK) and freeze killed (FK) trophozoites were prepared as other controls for trophozoite killing. A combination of α-pinene and eucalyptol was found to have greater inhibitory effects than α-pinene alone (*; $P = 0.015$).

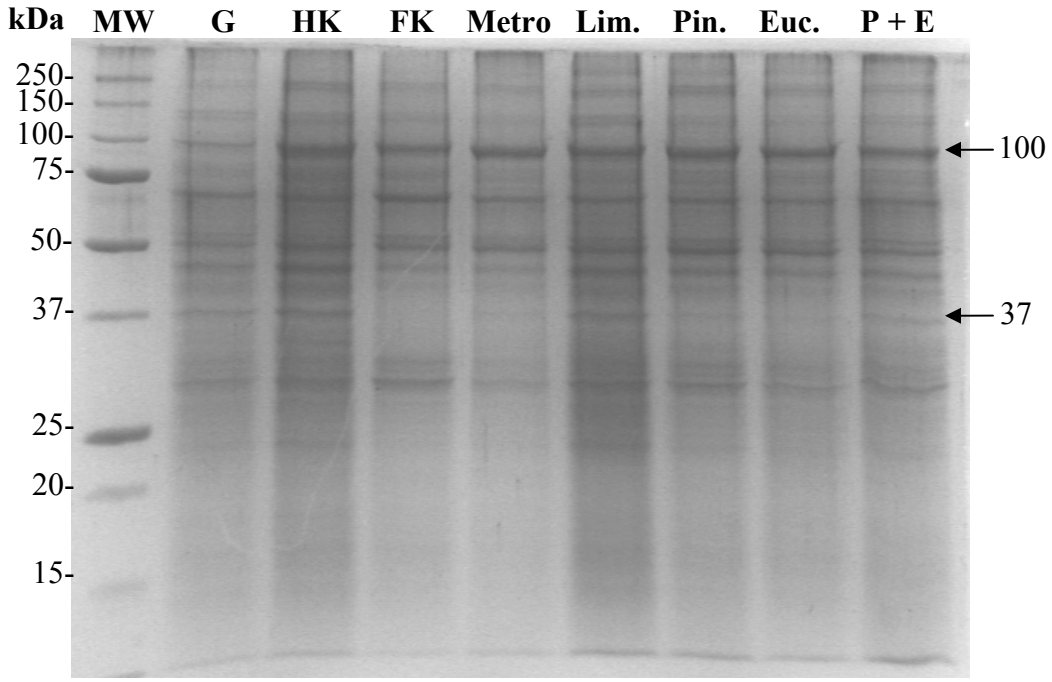


Figure 2.16 The effect of plant oil constituents on *G. duodenalis* trophozoite protein profile using SDS-PAGE analysis.
G. duodenalis trophozoites (**G**; 5 x 10^7 ml^{-1}) were incubated in the presence or absence of final concentrations of 20 µg metronidazole (**Metro.**) and either 0.01% limonene (**Lim.**), 0.01% α-pinene (**Pin.**), 0.0025% eucalyptol (**Euc.**) or a combination of α-pinene and eucalyptol (**P+E**) in 96 well microtitre plates and compared with untreated trophozoites (**G**). Heat killed (**HK**) and freeze killed (**FK**) trophozoite lysates were prepared as other controls for trophozoite killing. All lysates prepared were loaded on a 12% acrylamide separating gel and 4% acrylamide stacking gel and electrophoresed at 180V for 55 mins. Protein bands are visualised using the Colloidal blue G staining system and run against a molecular weight marker (**MW**; Bio-Rad).

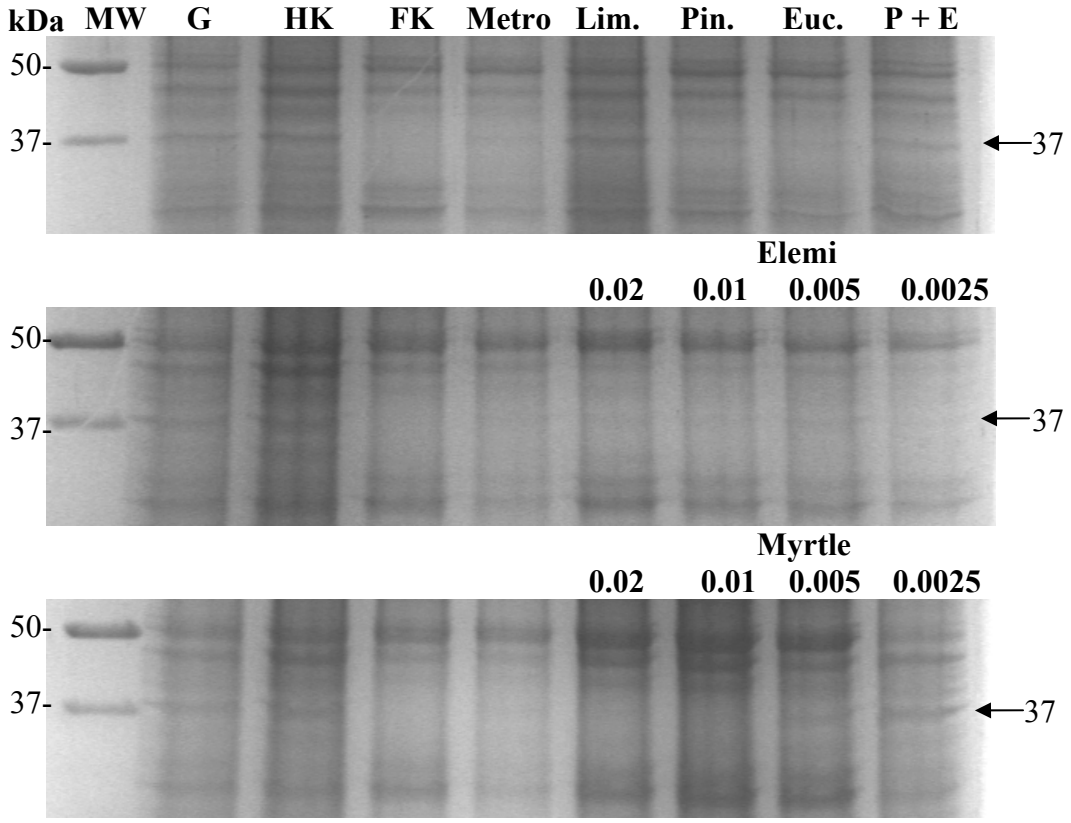


Figure 2.17 The effect of plant oils and their constituents on a 37 kDa *G. duodenalis* trophozoite protein using SDS-PAGE analysis.
G. duodenalis trophozoites (**G**; 5 x 10^7 ml^{-1}) were incubated in the presence or absence of final concentrations of 20 µg metronidazole (**Metro**.) and either 0.01% limonene (**Lim**.), 0.01% α-pinene (**Pin**.), 0.0025% eucalyptol (**Euc**.) or a combination of α-pinene and eucalyptol (**P+E**) and 0.0025 – 0.02% elemi oil or myrtle in 96 well microtitre plates and compared with untreated trophozoites (**G**). Heat killed (**HK**) and freeze killed (**FK**) trophozoite lysates were prepared as other controls for trophozoite killing. All lysates prepared were loaded on a 12% acrylamide separating gel and 4% acrylamide stacking gel and electrophoresed at 180V for 55 mins. Protein bands are visualised using the Colloidal blue G staining system and run against a molecular weight marker (**MW**; Bio-Rad).

Table 2.5 Quantity One software analysis of the intensity of a 37 kDa protein band

A. Elemi oil.

	Giardia	0.02	0.01	0.005	0.0025
Band Intensity	**6.11**	**0.00**	**0.00**	**0.00**	**0.00**

B. Myrtle oil.

	Giardia	0.02	0.01	0.005	0.0025
Band Intensity	**12.78**	**0.00**	**0.00**	**8.89**	**16.78**

C. Limonene, α-pinene, eucalyptol, or a combination of α-pinene and eucalyptol.

	Giardia	Limonene	α-pinene	Eucalyptol	P + E
Band Intensity	**7.78**	**7.34**	**3.89**	**0.45**	**3.45**

Computer software image analysis was performed on selected SDS-PAGE gels shown in Figure 2.17. Analysis of the 37 kDa protein band using the Quantity One software package (Bio-Rad) highlighted the intensity of this band when *G. duodenalis* trophozoites were incubated with **A**) 0.0025 – 0.02% elemi oil, **B**) 0.0025 – 0.02% myrtle oil and **C**) either 0.01% limonene, 0.01% α-pinene, 0.0025% eucalyptol or a combination of α-pinene and eucalyptol.

2:5 DISCUSSION

These experiments were designed to determine working concentrations of *G. duodenalis* trophozoites, diluents and reference drug for use in this project. Also determined were the effect of various PEOs and their main constituents on the viability and cell biology of *Giardia* trophozoites. Light microscopy was used identify morphological changes in these parasites and to determine trophozoite survival using Trypan blue inclusion / exclusion coupled with motility and morphology. The Trypan blue dye exclusion assay is the most commonly utilized test for cell viability (Eisenbrand *et al*. 2002; Freshney 1994; Patterson 1979; Pappenheimer 1917). The usefulness of this procedure is limited since the number of blue-staining cells increases following addition of the dye, requiring that cells be counted within 3 – 5 min (Hudson and Hay 1980). The proposed concentration of diluent to be used for the solublisation of PEOs was 75% diluent to 25% plant essential oils (PEOs) on a volume: volume ratio, to create a stock which would then be diluted further with medium. This stock concentrate would then give a volume of diluted PEO which could be pipetted with accuracy. A reference drug is commonly used, so that an unknown compound can be compared with a compound of known efficacy. In the case of these experiments, metronidazole, as the recognised drug of choice for giardiasis, was used.

PEOs influence the cell biology of *G. duodenalis* trophozoites over time. PEOs caused the swelling, distortion, blebbing and increased killing of trophozoites *in vitro*. Incubation of *G. duodenalis* trophozoites with PEOs at a final concentration in the well of 0.02% significantly reduced their viability over the 24 h period studied. All PEOs were equally effective at this concentration, inhibiting the viability of trophozoites by 100% (Appendix 6).

Recent advances in the understanding of the biochemistry and molecular biology of protozoa have permitted the study of molecules that directly participate in the parasites life cycle or their pathogenicity (North, Mottram and Coombs 1990). In this series of experiments, the mechanisms by which plant oils and their main constituents exerted an antigiardial effect were examined using SDS-PAGE analysis.

This study shows that the plant oils effects and to a limited extent, their main constituents can be modified at the molecular level. As well as causing morphological changes, PEOs can alter the protein profile of *G. duodenalis* trophozoites, by which the reduction of viability possibly occurs.

Incubation of *G. duodenalis* trophozoites in DMSO alone at concentrations found when used as a diluent for PEOs caused a significant reduction in their viability (Table 2.2, $P < 0.05$). Damage caused by DMSO to trophozoites ranged from the retention of typical pyriform morphology but with Trypan blue uptake, to parasites being rounded and swollen, again with Trypan blue inclusion (Figure 2.1). This is at odds with previous findings for DMSO where concentrations of up to 10% are routinely used for cryopreservation with little or no effect (Phillips, Boreham and Shepard 1984) and concentrations of up to 1% having been used *in vitro* for drug dilutions having no effect on trophozoite morphology or viability (Müller *et al.* 2007; Céu Sousa 2001). As high error levels were recorded in the experiments performed and, due to variability of these results, it was decided that DMSO would not be used as a diluent for PEOs.

Metronidazole was chosen as the reference drug and a positive control for inhibition of viability due to its current use in the treatment of giardiasis. This drug has known mechanisms of action, with its effect on *G. duodenalis* trophozoites being due to the generation of nitro radicals through reduction of the nitro ring by ferredoxin, which is in turn reduced by pyruvate:ferredoxin oxidoreductase (PFOR) (Samuelson 1999; Townson *et al*. 1994; Upcroft 1998; Upcroft *et al*. 1999). Reduced metronidazole serves as a terminal electron acceptor which binds covalently to DNA macromolecules (Müller 1983). This results in DNA damage in the form of a loss of helical structure, impaired template function and strand breakage, with subsequent trophozoite death (Gillis & Wiseman 1996; Townson *et al*. 1994). In addition to this effect, metronidazole inhibits trophozoite respiration (Farthing 1992; Paget *et al*. 1989).

Using microscopic evaluation of parasite morphology and mobility, Jokipii and Jokipii first demonstrated that metronidazole and tinidazole were effective (Jokipii & Jokipii 1980). Subsequently, morphology (Meloni *et al*. 1990) growth inhibition (Crouch *et al*. 1990; Edlind 1989; Majewska *et al*. 1991), [^{3}H] thymidine incorporation (Inge & Farthing 1987; McIntyre *et al*. 1986), serum killing (Hill, Burge and Pearson 1984), vital dye exclusion (Hill, Burge and Pearson 1984; Thiriat, Sidaner and Schwartzbrod 1998), inhibition of adherence (Baveja, Bhatia and Warhurst 1998; Farbey, Reynoldson and Thomson 1995; Meloni *et al*. 1990), metabolic (Céu Sousa & Poiares-da-Silva 1999) and colorimetric (Kang *et al*. 1998) assays have been employed to measure the *in vitro* response of the parasite to many therapeutic agents. However, as indicated by the variety of assays used, there is no standard for *in vitro* testing, making it difficult to compare results and apply *in vitro* findings to the clinical setting (reviewed by Gardner & Hill 2001).

Treatment of trophozoites in experiments with different concentrations of metronidazole produces reduced trophozoite viability in a dose dependant manner (Figure 2.2). Although all trophozoites were killed after 24 h exposure to 67 μg ml^{-1} metronidazole, concentrations of up to half that caused > 95% trophozoite death. The parasites used in this project were the kind donation of Dr. T. Paget and it was discovered that the MIC of metronidazole observed (67 μg ml^{-1}) was 4 times greater than that observed in his own laboratory (T. Paget, Personal communications 2005). However, by investigating the 50% and 90% inhibition concentration levels it was demonstrated that metronidazole activity was present at low concentrations (IC_{90} = 21.8 μg ml^{-1} and IC_{50} = 5.6 μg ml^{-1}; Table 2.3). This brings the level of activity of metronidazole on the stock of *G. duodenalis* trophozoites used in the SPDL to activity levels found with Dr. Paget's laboratory stocks.

This variability of the effects of metronidazole on different clones and isolates of *G. duodenalis* has been shown previously and susceptibility was also shown to vary in culture with susceptible strains becoming slightly more resistant and increasing susceptibility in resistant strains (Argüello-García *et al*. 2004). Resistance to metronidazole has been induced *in vitro* (Boreham, Phillips and Shepard 1988) and

correlates with decreased activity of parasite pyruvate: ferredoxin oxidoreductase (PFOR), which is required for reductive activation of nitroimidazoles (Upcroft & Upcroft 1993). In the past, susceptibility assays have been monitored for a variety of end points and reported as 50% inhibitory concentrations (IC_{50}) for *Giardia* ranging from 0.01 to 1 μg ml^{-1} (Upcroft & Upcroft 1993), 2.38 – 11.5 μM (Cruz *et al*. 2003), 50% inhibitory dose (ID_{50}) values of 0.2 and 1.5 μg ml^{-1} (Upcroft & Upcroft 1993), minimum lethal concentrations (MLCs) after 72 h of 50 to 100 μM for susceptible organisms (Upcroft *et al*. 1999).

Trypan blue inclusion in metronidazole-treated trophozoites showed enlargement of both nuclei and strong staining of the edges of the nuclei (Figure 2.3 A). Also shown was evidence of blebbing and cytoplasmic granulation / condensation suggestive of apoptosis; an intriguing proposition as the cornerstone for apoptosis, the mitochondria, is absent in this protist (Chose *et al*. 2003). This formation of blebs or giant membrane protrusions has also been observed by Pérez-Arriaga *et al*. (2006) where the authors treated trophozoites with varying concentrations of curcumin, a naturally formed compound from the food spice, turmeric. In the experiments performed with myrrh, myrtle and elemi oils, giant protrusions were formed in many of the trophozoites (Figure 2.3 C, D and E). In all cases, condensation of the cytoplasm was evident, showing as granular staining with large vacuoles also formed in the cytoplasm of myrtle oil-treated trophozoites (Figure 2.3 D).

The formation of giant membrane protrusions may or may not be a part of some kind of programmed cell death, it may also be due to changes to the structure of the cell membrane caused by the PEOs or their constituents. Changes in the osmoregulation of the cell could also be indicated by the swelling and rounding up of the trophozoites Figure 2.3 B, D and E) which was also observed by Pérez-Arriaga *et al*. (2006) in curcumin treated trophozoites and Campanati & Monteiro-Leal (2002) with metronidazole treated trophozoites. Interaction of the PEO with the cellular membrane is quite possible due to the lipophilic properties of PEOs and their ready diffusion across cell membranes (Boyom *et al*. 2003). Large vacuole formation in myrtle treated trophozoites (Figure 2.3 D) indicates that some kind of programmed

cell death may have been initiated, as this has also been observed in curcumin-treated trophozoites (Pérez-Arriaga *et al*. 2006).

In order to elucidate MIC for each PEO used, a titration was carried from 0.02% to 0.005% in the first instance and then down to 0.00125% for those oils whose MIC were determined to be < 0.005%. Sweet fennel, lavender, amyris, patchouli and buchu oil were determined to have n MIC of 0.02%, while marjoram, palmarosa, thyme and geranium all had a MIC of 0.01%. Only the oils of myrrh, myrtle and elemi were able to kill 100% of trophozoites *in vitro* at a concentration of 0.005% (Figure 2.4 and Table 2.3). However, at concentrations lower than this, the efficacy of these oils was lessened with 68% of trophozoites remaining viable for myrtle and elemi oils at a concentration of 0.0025% (Figure 2.5). The efficacy was improved with the treatment of myrrh with 18% of trophozoites remaining viable with 0.0025% myrrh and 62% remaining viable below a myrrh concentration of 0.00125% (Figure 2.5). At this lower concentration of 0.00125% both myrtle and elemi oils had 78% and 80% of viable trophozoites after 24 h incubation *in vitro*, respectively. This was significantly greater than with myrrh (Figure 2.5, $P < 0.05$). From this information it can be seen that myrrh oil has the greatest antigiardial effects causing a significant reduction in trophozoite viability at 0.0025%. Whilst this concentration was not the MIC, the reduction was such that possibly of longer incubation might cause a further lowering of the MIC of 0.005% at 24 h to perhaps 0.0025% at 48 or 72 h.

As with the metronidazole titration, the IC_{90} and IC_{50} levels for the oils tested were calculated. There was great variation between the IC_{90} and IC_{50} activities of the PEOs which agreed with their variable MIC activity, producing a range of values (IC_{90} = 0.003 – 0.013 % [30 – 130 μg ml^{-1}], IC_{50} = 0.0009 – 0.007 % [9 – 70 μg ml^{-1}], Table 2.3) where 0.00001% = 1 ppm = 1 μg ml^{-1}. Oils of myrrh, myrtle and elemi at their MIC of 0.005% (50 μg ml^{-1}) were found to be more active than metronidazole at its determined MIC of 67 μg ml^{-1} indicating that PEOs may well be an effective alternative to conventional chemotherapeutics. However, there is a very sharp decline in PEO activity at lower than 100% inhibitory concentrations compared

with metronidazole, with the conventional drug being more effective at lower concentrations than the PEOs. This suggests that there is a narrower window of activity for PEOs.

It is possible to identify the composition of oils, generally using methods such as Gas Chromatography (GC) coupled with Mass Spectroscopy (MS). This provides an insight into the potentially active constituent(s) that could be giardicidal *in vitro*. Investigations into the biological activities of essential oils of some medicinal plant species have revealed that some of them exhibited interesting activities, such as antibacterial (Lemos *et al*. 1990; Ferdous *et al*. 1992; Faouzia, Souad and Tantaoui-Elaraki 1993; Demetzos *et al*. 1997; Fyfe, Armstrong and Stewart 1997; Smith-Palmer, Stewart and Fyfe 1998 & 2002; Hammer, Carson and Riley 1999; Burt 2004), antifungal (Hammer, Carson and Riley 2003; Terzi *et al*. 2007) and antiplasmodial activity (Benoit-Vical *et al*. 1999 & 2001). In past studies, the whole oil has been investigated for any *in vitro* activity, with the composition of the oil being investigated afterwards, in order to determine any possible active constituent. Investigations into the active principles of essential oils having antibacterial or antifungal activity has been carried out (Hinou, Harvala and Hinou 1989; Oloke 1992; Hammerschmidt *et al*. 1993; Zakarya, Fkih-Tetouani and Hajji 1993 and Carson & Riley 1995).

Using GC-MS information supplied by F.D. Copeland & Sons (Appendix 3) the compositions of the oils myrrh, myrtle and elemi were determined and the main constituents (i.e. those found in the greatest quantities in the oil) were identified. In the case of myrtle oil, the major constituents were the monoterpenes α-pinene and eucalyptol (syn. 1,8-cineole) comprising 51.72% and 22.35% of the whole oil, respectively. Elemi oil contained mostly the terpene limonene (syn. (+)-*p*-Mentha-1,8-diene; (+)-Carvene; (*R*)-4-Isopropenyl-1-methyl-1-cyclohexene) (51.54%), α-phellandrene (16.23%) and elemol (10.90%). Myrrh oil comprised mainly of the furanic sesquiterpenes, furanodiene (31.97%) and curezene (26.69%). At the time of writing, only α-pinene, eucalyptol and limonene were readily available from chemical companies and, therefore, only experiments concerning myrtle and elemi oils could be carried out.

Using this information, investigations were carried out to determine which constituent was the active ingredient(s) of the whole oil, responsible for the antigiardial activity. The first experiments used the constituents at concentrations equivalent to those found in the whole oil at the screening concentration of 0.02%. When used at these concentrations the main constituent of myrtle oil, α-pinene, completely reduced the viability of trophozoites to zero. Its next most abundant constituent was eucalyptol which reduced the viability of trophozoites to 83% ± 5% and a combination of these 2 constituents completely inhibited trophozoite viability (Figure 2.6).

The only constituent from elemi oil available for study was limonene and this constituent was responsible for the complete inhibition of trophozoite viability. These results indicate that α-pinene is the active constituent in myrtle oil and limonene is the active constituent in elemi oil. When the constituents were used at concentrations equivalent to the oils MIC (0.005%) a different efficacy emerged. The viability of trophozoites incubated with these constituents singly or in combination was unaffected when compared with untreated controls (Figure 2.7). This suggested that the compounds present in the greatest proportions are not necessarily responsible for their antigiardial activity. There may be a lower limit to the efficacy of these constituents where combinations of all constituents of the oils act in synergy to kill *G. duodenalis* trophozoites at this low MIC of 0.005%.

This activity of constituents is somewhat similar to the findings of Cimanga *et al*. (2002) where no correlation between the amount of major constituents such as eucalyptol, α-pinene, *p*-cymene, cryptone or thymol and antibacterial activity was observed from 15 medicinal Congolese plants and their essential oils. Some of the constituents in that study, such as limonene, *p*-cymene and α-pinene, had been previously shown to exhibit low levels of antibacterial activity (Knobloch *et al*. 1989 and Chalchat *et al*. 2000).

Apart from antibacterial activity, limonene also has antifungal activity, inhibiting the growth of *Fusarium verticillioides* MRC 826 *in vitro* (Dambolena *et al*. 2008) and

has shown some antiparasitic actions as well, being able to inhibit the development *in vitro* of intraerythrocytic stages of *P. falciparum* (Moura *et al*. 2001; Rodrigues Goulart *et al*. 2004). Antifungal actions have been observed with eucalyptol (Hammer, Carson and Riley 2003; Terzi 2007) and antibacterial activity was also evident (Hammer, Carson and Riley 1999; Papadopoulos *et al*. 2006).

The speed at which all the oils showed activity against *G. duodenalis* trophozoites was very rapid. Four of the 12 oils tested were able to completely reduce the viability of trophozoites to 0% in only 30 min of incubation *in vitro* (Figure 2.8). These oils were lavender (MIC = 0.02%), marjoram (MIC = 0.01%), palmarosa (MIC = 0.01%) and geranium (MIC = 0.01%). All other oils tested, with the exception of myrrh, were able to kill all trophozoites within 60 min of incubation. This is significantly faster than metronidazole which, after 120 min of incubation, still had no discernable effect on trophozoites (Figures 2.8 and 2.9). With metronidazole complete inhibition of trophozoites occurs somewhere between 2 h and 24 h of incubation.

For adults, a standard treatment (up to 2 g metronidazole daily for between 3 and 7 days) delivers a peak plasma concentration of 40 $\mu g\ ml^{-1}$ within 3 h (McEvoy 1995). Ileal and colonic concentrations reach between 70 – 80% of the dose (Haughton *et al*. 1979). Metronidazole has a half-life of approximately 8 h in humans, with 60 – 80% of the drug and its metabolites excreted in the urine and only 6 – 15% excreted in faeces. This means that a constant 'top up' of drug is required to kill trophozoites *in vivo*. This also means that there is a greater risk of any side effects occurring, reduced patient compliance with the treatment regimen and increased incidence of drug resistance in the parasite.

A drug which can be fast acting and with a greater retention time within the target area (in the case of *G. duodenalis* the small intestine) would be of great benefit in reducing the quantity of drug to be taken whilst maintaining the same, if not increased, efficacy. Data on the bioavailability of plant oils or their constituents are, however, scarce. One class of constituent is the flavonoids. There have been over 4000 different flavonoids described, categorized into flavonols, flavones, catechins,

flavanones, anthocyanidins and isoflavonoids. Little information is available, but the anthocyanins have been demonstrated to have low serum bioavailability in humans, but considerable longevity in the gastrointestinal tract and are known to reach the colon largely undegraded (up to 85% of the initial dose) either following their consumption (Kahle *et al*. 2006) or following *in vitro* simulation of human digestion (McDougall *et al*. 2005). This makes them ideal candidates for a potential drug with antigiardial action.

However, the anthocyanidins are just one of a multitude of different constituents found in the PEOs tested and information on the bioavailability of terpene compounds (found within myrtle and elemi oils) is scanty. Further work would need to be carried out in this field before one could properly determine their suitability as a novel antigiardial drug. Nevertheless, the rapid action of the PEOs used in this study has shown a potential for novel drug design which could reduce the duration of treatment.

Whilst the recognised drug for giardiasis, metronidazole, was used as a control for killing, different methods were also used in SDS-PAGE experiments to show any clear differences or similarities in protein profiles when compared to oil/constituent treated trophozoites. For this purpose both heat killing (56°C for 10 min) and freeze killing (liquid nitrogen, 2 min) were employed. Only one cycle of freeze thawing was used as several such cycles are generally required for the lysis of trophozoites. Following one cycle of rapid freezing and thawing or after heat killing, trophozoites were non-viable, being round and swollen and including Trypan blue.

The use of higher concentrations of parasites did have an effect on PEO treatment. In both oils (myrtle and elemi) a higher MIC was observed with 5×10^7 ml^{-1} than with 2.67×10^5 ml^{-1} trophozoites. Whilst the reduction of inhibition at the previously determined MIC of 0.005% for both oils was slight (2.4 ± 2.2% for myrtle and 3.1 ± 3.4% for elemi), it was found to be significant (Table 2.4, $P < 0.05$). This may have ramifications in the possible use of these oils for the treatment of giardiasis. In the lumen of the small intestine, the concentration of trophozoites can be high and a

giardiasis patient can shed 1×10^8 viable cysts per gram of faecal material (Roxström-Lindquist *et al*. 2006).

Myrtle and elemi oil have been shown to produce morphological changes in *Giardia* including trophozoite swelling, rounding, blebbing and large vacuole formation. Also shown was the speed at which viability is reduced by these oils (60 min; Figure 2.8). The use of SDS-PAGE analysis can highlight any inhibition or upregulation of protein expression. The identification of these proteins may help in the understanding of how the PEOs or their constituents affect trophozoite viability.

The apparent upregulation of a ~100 kDa protein compared to untreated trophozoites was shown in all trophozoite treatments *in vitro*. This would strongly suggest that this protein is linked to mortality of the cell, whether it is some kind of 'programmed cell death' protein or associated with another means of death remains to be seen. Molecules such as the cysteine proteases (caspases) have been shown to be a part of apoptosis in various eukaryotic cells and can be found as part of the mitochondria (Chose *et al*. 2003). However their presence in organisms which are devoid of mitochondria such as *G. duodenalis* and *Trichomonas vaginalis* remain unclear. To date, sequence analyses for homologues of mammalian apoptotic genes or proteins have not been reported in protozoa, especially *Giardia* (Nasirudeen 2005; Chose *et al*. 2003a; Chose *et al*. 2003b; McArthur *et al*. 2000). This is not to say that a caspase-like apoptosis reaction does not occur, or that caspases (or caspase-like proteins) are not present. Chose *et al*. (2002) has shown that with *T. vaginalis*, drugs which are caspase activators were able to induce an apoptosis-like action and that inhibitors of caspases prevented such apoptosis. In the same paper and in subsequent papers (Chose *et al*. 2003a; Chose *et al*. 2003b), this particular author also claims to have shown the same apoptotic-like morphological changes in *Giardia* using apoptosis inducing drugs, although these data have not been formally published.

Both a 37 kDa and a ~70 kDa protein (present in untreated trophozoites) were abolished by high concentrations of myrtle oil (0.02 and 0.01%) and started to reappear when lower concentrations were used (0.005 and 0.0025%). The 37 kDa

protein was also abolished in elemi oil treated trophozoites in the manner described for myrtle and was also absent in freeze-killed and metronidazole-killed parasites, but not in heat-killed parasites.

This 37 kDa protein ablated by the whole oils reappears when trophozoites were incubated with either α-pinene or limonene. Eucalyptol did not abolish this band completely. When investigating band intensities using the Quantity One software the intensity of the 37 kDa band present following limonene treatment is similar to that found in untreated controls and treatment with α-pinene reduces the band intensity by almost 50% (Table 2.5 C) with eucalyptol treatment causing the intensity of the band to be reduced to background levels. This result shows that whilst elemi oil can abolish the 37 kDa protein at low concentrations (0.0025%) this is not due to its main constituent, limonene. Similarly, eucalyptol and not α-pinene is responsible for the same action in myrtle oil.

Many important parasite species such as *E. histolytica*, *T. cruzi*, *Leishmania* spp., *T. vaginalis* and *Plasmodium* spp. contain multiple proteases that play a role in their life cycle, morphogenesis and infectivity (McKerrow *et al*. 1993). Whilst both myrtle and elemi oil can cause morphological changes to *G. duodenalis* trophozoites *in vitro* without disruption of the cell, the speed at which those changes occurred suggested damage at the molecular level, possibly by inducing a form of programmed cell death.

An understanding of what the proteins affected by the oils are and how they might be involved in the survival of the parasite may lead to a more defined mode of action for theses oils. Further analysis of the molecular weights of proteins mentioned here, especially the 37 kDa protein, is required. Without knowing the specific molecular weight and, preferably the amino acid sequence of the proteins highlighted in this study it is difficult to determine what role they have to play in the PEO induced death of *Giardia* trophozoites. The use of techniques such as Western blotting, 2-D electrophoresis and sequence analysis of the *Giardia* genome should allow the

correct identification of the proteins affected by the PEOs. Identifying these proteins would allow a better understanding of the mechanism of action of the PEOs tested.

To the author's knowledge this is the first time that such an extensive screening of PEOs has been carried out on the *Giardia* parasite. This study has shown that PEOs were effective in inhibiting trophozoite viability over a 24 h incubation period at a final concentration in the microtitre well of 0.02%. Furthermore, the parasite was found to be most sensitive to the oils of myrrh, myrtle and elemi with an MIC for these PEOs being 0.005%. Antigiardial activities of PEOs were found to be rapid with all but myrrh acting within 60 min of incubation. Further analysis of the PEOs suggests that the monoterpenes, α-pinene from myrtle oil and limonene from elemi oil were the active constituents responsible for the killing of *G. duodenalis* trophozoites *in vitro* found within these oils.

CHAPTER 3

Cryptosporidium parvum

Increased spontaneous excystation of *Cryptosporidium parvum* oocysts over time following pre-treatment with PEOs and their constituents.

3:1 ABSTRACT

Interventions in the *Cryptosporidium* life cycle can, if they reduce the ability of the extracellular stages to infect enterocytes and thus reduce the number of intracellular stages, become potentially effective treatments for cryptosporidiosis. The term 'spontaneous' excystation is used to define the process of excystation *in vitro* that occurs in the absence of known triggers, apart from temperature. This distinguishes it from the excystation that occurs following treatment with the maximised *in vitro* excystation protocol of Robertson, Campbell and Smith (1993b). In this study, 12 PEOs were investigated for anticryptosporidial activity. When *C. parvum* oocysts were incubated for 24 h in the presence of 0.2% PEOs spontaneous excystation was increased, compared to untreated oocysts. Oils of sweet fennel, geranium and palmarosa caused significant increases in the spontaneous excystation rate when compared to untreated control oocysts (77.1 ± 12%, 90.9 ± 1.1%, 96.9 ± 1.7%, respectively; $P < 0.01$ for all oils). The effect of these oils main constituents, *trans*-anethole, citronellol and geraniols were used to investigate the spontaneous excystation rate of *C. parvum* oocysts *in vitro*. These constituents had activity levels consistent with the effect of the whole oil (sweet fennel = 84.6 ± 5.7% *vs*. *trans*-anethole = 87.9 ± 2.5%; geranium = 91.0 ± 1.2% *vs*. citronellol = 87.6 ± 2.7%; palmarosa = 96.8 ± 1.5% *vs*. geraniol = 97.8 ± 1.4%) during incubation at 37°C for 24 h. Spontaneous excystation occurred in a time dependent manner for all PEOs and constituents tested. The most rapid increase in spontaneous excystation occurred with sweet fennel and *trans*-anethole after 4 h of incubation (sweet fennel = 0.4 ± 0.5% to 57.1 ± 2.5%; *trans*-anethole = 0.4 ± 0.5% to 60.9 ± 1.1%, untreated controls = 18.7 ± 2.3%). PEOs influence the *in vitro* excystation of *C. parvum* oocysts in a temperature and time dependent manner, but in the absence of known triggers such as bile salts, reducing agents and proteases. It was clear that the main constituents of the oils were the causative agents of spontaneous excystation induction and that this was also time dependent. Whilst aromatic ethers had a more rapid response, terpenes had a more sustained and ultimately greater effect on spontaneous excystation. Possible uses for these attributes are discussed.

3:2 INTRODUCTION

The enteric parasite, *Cryptosporidium parvum*, causes self limiting diarrhoea in immunocompetent hosts and chronic, severe diarrhoea with potentially fatal consequences in immunocompromised hosts (Hunter & Nichols 2002). Of the 19 species and over 40 genotypes of the protozoan parasite *Cryptosporidium*, 8 species and 5 genotypes are known to infect humans and of these, 2 species (*C. hominis* and *C. parvum*) are responsible for the majority of human disease (Smith 2008; Smith *et al*. 2007; Feltus *et al*. 2006; Leoni *et al*. 2006; Nichols, Campbell and Smith 2006; Caccio *et al*. 2005; Ryan *et al*. 2004; Xiao *et al*. 2004). *C. parvum* is the species most studied with respect to infectivity *in vivo*, viability *in vitro* and as it can be cultured *in vitro*, is also used mostly for drug discovery investigations.

In the immunocompetent host, *Cryptosporidium* infection leads to self limiting diarrhoea after 2 – 3 weeks. This can be uncomfortable for the infected individual and can have financial implications if they cannot work. In the developed world this can be perceived to be an inconvenience. However, in developing countries this may affect an entire household if the main income earner is unable to work.

When this parasite infects an immunocompromised host the diarrhoea may not be self limiting and can become chronic with potentially fatal consequences (Hunter & Nichols 2002). This may also be true for otherwise healthy young children or the very old.

Of over 200 drugs tested, only nitazoxanide has been demonstrated to have efficacy for cryptosporidiosis with immunocompetent patients. It inhibits *C. parvum* growth *in vitro* and has been licenced in the USA by the Food and Drug Administration (FDA) since June 2004 for use in all persons ≥ 1 year of age (Rossignol 2006; Bailey & Erramousepe 2004; Smith & Corcoran 2004; Amadi *et al*. 2002; Theodos *et al*. 1998).

Oocysts of *Cryptosporidium* occur in the aquatic environment throughout the world and it is through the drinking of contaminated water and then person to person

transmission that most infections are acquired. The greatest such outbreak occurred in Milwaukee, USA in which over 400,000 individuals were estimated to have been infected (MacKenzie *et al*. 1994a and 1994b). Faecal contamination from infected animals is the source of oocysts (Hansen & Ongerth 1991; LeChevallier, Norton and Lee 1991). Oocysts are remarkably robust being able to persist in water for several months depending on the temperature of the water (Robertson, Campbell and Smith 1992; Chauret *et al*. 1995; Olson *et al*. 1999) and are extremely resistant to the disinfectants commonly used in drinking-water treatment such as chlorine and ozone (Korich *et al*. 1990; Finch *et al*. 1993). Characteristics of this organism such as environmental robustness and low numbers required for infectivity (Dupont *et al*. 1995; Okhuysen *et al*. 1999) make it one of the most critical pathogens in the production of safe drinking-water from surface water.

In vitro and *in vivo* investigations into *C. parvum* excystation have highlighted some of the host- and parasite-derived triggers required to initiate infection. These include temperature (37^{o}C), pH fluctuations, bile salts, reducing agents, proteases and time (Widmer *et al*. 2007; Smith, Nichols and Grimason 2005; Kato *et al.* 2001; Robertson, Campbell and Smith 1993b; Fayer & Leek 1984).

The maximised *in vitro* excystation method of Robertson, Campbell and Smith (1993b), use these host derived triggers to mimic transit through the acidic stomach to the alkaline small intestine (Smith, Nichols and Grimason 2005; Kato *et al.* 2001; Robertson, Campbell and Smith 1993b; Fayer & Leek 1984). This is a recognised rapid and simple means of determining the ability of sporozoites to undertake the first step of the infection process. Temperature (37^{o}C) and time (up to 24 h) are major triggers for maximised *in vitro* excystation (Robertson, Campbell and Smith 1993b; Smith, Nichols and Grimason 2005).

Exposure to acid (pH ~2) followed by incubation in bile salts, reducing agents and proteases, mimic transit through the acidic stomach to the alkaline small intestine and enhance excystation *in vitro*. The fact that oocysts can excyst *in vivo* and cause

disease in extraintestinal locations indicates that some of these host-derived triggers are not essential (Smith, Nichols and Grimason 2005).

C. parvum oocysts are known to excyst within the small intestine of the mammalian host and their sporozoites multiply in the epithelial enterocytes. To this end, the parasite has adapted to passage through the acidic environment of the stomach without excysting before reaching the site of infection. Studies by Widmer *et al*. (2007) investigated this phenomenon and demonstrated that *C. parvum* oocysts are unaffected by incubation for 1 h in HCl at 37°C and that temperature alone cannot cause the excystation of the oocysts. However, passage through the stomach can take up to 4 h and the effect of acid for this length of time on *C. parvum* oocysts was not determined, nor was the effect that temperature had upon *C. parvum* excystation after acidification.

Excystation of *C. parvum* oocysts in the stomach, which is too acidic for sporozoite survival (optimal pH 6.2, Woodmansee *et al.* 1987), causes sporozoite lysis, reduces enterocyte invasion and the subsequent propagation of infection. Similarly, stimulating oocyst excystation in the intestinal tract of the infected host before they are excreted reduces the number of infectious oocysts in the environment and limits the potential for propagation of infection.

Interventions in the *Cryptosporidium* life cycle that reduce the ability of extracellular stages to infect enterocytes, by reducing the number of intracellular stages, can become potentially effective treatments for cryptosporidiosis. As sporozoites are the first extracellular stages generated in the infected host, interventions that influence the timing of their release from intact oocysts, including excystation in abnormal sites or ablation of excystation can be considered potentially therapeutic. By modifying the processes by which oocysts excyst we may be able to influence the propagation of infection. The isolate of *C. parvum* and how it has been purified may also influence the *in vitro* investigations of excystation in this parasite.

The antimicrobial properties of herbs and spices have been recognised for thousands of years and recently there has been renewed interested in such products, especially PEOs (Smith-Palmer, Stewart and Fyfe 1998; Kumar & Berwal 1998). Only 2 plant extracts have been tested against *Cryptosporidium* with any success. A mild (22.4%) reduction in *C. baileyi* oocyst output in infected chickens was observed when chicken were given garlic extract. Its effect was comparable to 2 commercially available derivatives of an anticoccidial drug (triazinone) (Sreter, Szell and Varga 1999). When an extract of pine bark was given to *C. parvum* infected immunosuppressed C57BL/6N mice, oocyst shedding was reduced significantly, although the pine bark extract failed to decrease parasite colonisation of intestinal tissue (Kim & Healey 2001).

One possible target for any novel drug is to affect the excystation of *Cryptosporidium* oocysts. Whilst some of the host and parasite-derived triggers for excystation are known (Smith, Nichols and Grimason 2005; Kato *et al.* 2001; Robertson, Campbell and Smith 1993b; Fayer & Leek 1984), their precise involvement in the process is still being investigated. Interventions to the excystation process, whether it is enhancing or inhibitory, may provide clues as to the excystation process itself, or may lead to potential therapeutics.

In this study, the effect of pre-incubating intact *C. parvum* oocysts with acidified Hanks Balanced Salt Solution (HBSS; pH 2.75) for 1 – 4 h prior to incubation in non-acidified HBSS (~ pH 7) up to 24 h were assessed to determine whether acid influenced *C. parvum* excystation *in vitro*. As the maximised *in vitro* excystation method can determine the ability of sporozoites to undertake the first step of the infection process, the effect of incubating 12 different PEOs over 24 h with intact *C. parvum* oocysts was investigated to determine whether they influenced oocyst excystation dynamics *in vitro*. Of the 12 oils tested sweet fennel, geranium and palmarosa were found to have the greatest effect in increasing spontaneous excystation, without having any inhibitory effect on the normal excystation processes. These 3 oils were further investigated to determine their active

constituents and speed of activity. Some conclusions as to their possible uses are drawn.

3:3 MATERIALS AND METHODS

3:3.1 Oocyst preparations

Source of oocysts

C. parvum oocysts were purchased from Bunch Grass Farm (BGF), Idaho (IOWA isolate, Lots 06-30 and 28-27), USA and the Moredun Research Institute (MRI), Penicuik (MD isolate, Lot C1/07/01), UK. Isolates were stored in either phosphate buffered saline (PBS) for IOWA or Hanks balanced salt solution (HBSS) for MD isolate, containing 100µg of streptomycin ml^{-1} and 100U penicillin ml^{-1} at 5ºC until used. The IOWA isolate was passaged in neonatal calves and isolated by ether extraction using a modification of methods (discontinuous sucrose and caesium gradient centrifugation) described by Riggs and Perryman (1987). The MD isolate was passaged in neonatal lambs and isolated by acidification of faeces as described by Hill *et al.* (1990). Both isolates are *C. parvum* as determined by polymerase chain reaction, restriction fragment length polymorphism and DNA sequencing analyses at 2 18S rRNA loci (Xiao *et al*. 1999 & 2001; Nichols, Campbell and Smith 2003 & 2006) and the *Cryptosporidium* oocyst wall protein (COWP) locus (Homan *et al.* 1999) (data not shown).

Maximised in vitro excystation

Oocysts from the stock suspension were excysted *in vitro* at weekly intervals according to Robertson, Campbell and Smith (1993b) to determine the consistency of excystation throughout the study. Briefly, 100µl of stock suspension of *C. parvum* oocysts (2×10^7 ml^{-1}) were incubated in 900 µl acidified HBSS (Gibco, UK; pH 2.75, 60 min, 37ºC), then centrifuged (12,500 x ***g*** for 30 sec), the supernatant aspirated to 100 µl and the pellet resuspended in 1 ml HBSS. This was repeated twice and the washed pellet resuspended in 50 µl of 0.44% w/v sodium hydrocarbonate (BDH), 200 µl of 1% w/v bovine bile (Sigma-Aldrich, UK) in Hanks minimal essential medium (HMEM; Gibco, UK). Samples were incubated at 37ºC for 30 min and 4 h, with 10 µl aliquots viewed under Nomarski differential interference contrast (DIC) microscopy (Olympus BH2 microscope, x40 objective) at these time points. The percentage excystation (as an estimate of viability) was calculated according to Robertson, Campbell and Smith (1993b), whereby the proportion of totally excysted

(empty), partially excysted and intact oocysts were enumerated, in triplicate. Between 100 and 200 oocysts were counted for each enumeration and the percentage excystation was calculated as follows:

$$\frac{\text{No. of empty oocysts + No. of partially excysted oocysts}}{\text{Total no. of oocysts counted}} \times 100$$

The sporozoite ratio was also assessed at the same time and the total number of excysted sporozoites was enumerated and calculated as follows:

$$\frac{\text{No. of free sporozoites}}{\text{No. of empty oocysts + No. of partially excysted oocysts}} \times 100$$

A modified excystation protocol, that excluded the acidification step from the assay and which investigated the role that oocyst purification protocols have on excystation, was also employed and the above calculations performed. In all cases the number of empty oocysts present in the stock suspension was always subtracted from the number of empty oocysts enumerated post-excystation.

3:3.2 Experimental treatments of oocysts

PEO and constituent preparation

All PEOs were diluted in absolute ethanol to provide 25% v/v stock solutions (25% PEO, 75% absolute ethanol), which were used in these experiments. Complete information about each PEO used is given in Appendix 1. Briefly the oils used are as follows: sweet fennel, lavender, amyris, marjoram, palmarosa, patchouli, myrrh, myrtle, elemi, thyme, buchu and geranium. The most effective oils were determined to be sweet fennel (*Foeniculum vulgare* var. *dulce* BATT. & TRAB.), palmarosa (*Cymbopogon martinii* (ROXB.) J.F. WATSON) and geranium (*Pelargonium graveolens*). These oils were investigated with GC-MS to determine their composition and which constituents were in the highest concentrations. This procedure was carried out by F.D. Copeland & Sons with the composition of all the PEOs given in Appendix 3. Constituents which made the greater part of the PEO

were assessed at their equivalent concentration within the PEO, such that a constituent which comprises 75% of a PEO would be used at a concentration equal to 75% of the final concentration of the whole PEO, i.e. 75% of 0.2% = 0.15%. The major constituent of palmarosa oil is the monoterpene alcohol, geraniol (syn. 3,7-dimethyl-2,6-octadien-l-ol), comprising 80.02% of the whole oil. Sweet fennel oil is composed mainly of the aromatic ether, *trans*-anethole (syn. *trans*-1-methoxy-4-(prop-1-enyl) benzene) (68.33%). The major constituent of geranium oil was the monoterpene, citronellol (syn. 3,7-dimethyloct-6-en-1-ol) (39%). These constituents were used in experiments as previously described for PEOs.

The effect of temperature and time on spontaneous excystation in untreated oocysts

Stock, untreated oocysts were exposed to elevated temperature and time (37ºC, up to 24h), as both temperature elevation and time are known excystation triggers. A further temperature control consisted of stock, untreated oocysts incubated at 5ºC for 24 h. This process of excystation in the absence of all recognised triggers, apart from temperature was named 'spontaneous' excystation as opposed to excystation which requires known triggers including pH change, bile etc. (Robertson, Campbell and Smith 1993b; Smith, Nichols and Grimason 2005).

A 100 µl aliquot of *C. parvum* oocyst stock suspension (stock at 2 x 10^7 oocysts ml^{-1}) was incubated in 900 µl HBSS (final oocyst concentration = 2 x 10^6 ml^{-1}) for 4, 8, 12 or 24 h at 37ºC, prior to excystation using the maximised *in vitro* excystation protocol, at each of these time points.

The effect of 1 or 4 h acidification on the spontaneous excystation of C. parvum oocysts

100 µl of *C. parvum* oocyst stock suspension (stock at 2 x 10^7 oocysts ml^{-1}) was incubated in 900 µl acidified HBSS (pH 2.75) for either 1 or 4 h at 37ºC then washed as described previously. Subsequently, either i) the percentage excystation was determined under DIC microscopy or ii) oocysts were resuspended in 1 ml HBSS

and incubated for a further 1, 4 or 24 h at 37ºC to determine the effects of the 1 or 4 h acidification on oocyst spontaneous excystation.

Incubation of oocysts with PEOs

The following controls were used: a) 100µl of *C. parvum* oocyst stock suspension (stock at 2 x 10^7 oocysts ml^{-1}) mixed with 900 µl HBSS and incubated for 24h at 37ºC and b) an ethanol control, to determine whether ethanol affects *in vitro* excystation, consisting of 8 µl of a 75% v/v solution of absolute ethanol in distilled water, to produce a final concentration of 0.6%.

Eight µl of PEO diluted in ethanol (25% v/v stock) was added to a 1.5 ml microcentrifuge tube followed by 892 µl of HBSS and vortexing for 20 sec, followed by the addition of 100µl of a 2 x 10^7 ml^{-1} stock suspension of *C. parvum* oocysts (final concentration of 2 x 10^6 ml^{-1} oocysts suspended in 0.2% of PEO), vortexing and incubation for 24 h at 37ºC.

Determining whether PEO treatment influences maximised in vitro excystation when standard excystation triggers are used.

Following treatment with PEOs, each oocyst suspension was subjected to the maximised excystation protocol of Robertson, Campbell and Smith (1993b) as described previously, to determine whether PEO treatment had any effect on *in vitro* excystation when standard triggers were used. The rate of excystation of the remaining unexcysted oocysts from PEO treatment was determined.

Incubation of oocysts with PEO constituents

Having prepared the constituent stock in the manner described for PEOs, the volume of the stock added to a 1.5 ml microcentrifuge tube was adjusted to give the following final concentrations: a) geraniol 0.16%, b) *trans*-anethole 0.14% and c) citronellol 0.1%. This was followed by vortexing and addition of *C. parvum* oocysts as previously described for PEOs.

Incubation of C. parvum oocysts with PEOs and PEO constituents over a 24 h time course

A time course experiment was employed to determine how quickly the PEOs and their constituents caused spontaneous excystation. The PEOs and constituents were used at the same concentrations as in the 24 h experiments. The oocysts were incubated in the presence or absence of the PEO or PEO constituents for 4, 8, 12 and 24 h at 37ºC.

Following incubation, each sample was washed three times in HBSS and 10 µl samples were viewed under DIC and the percentage excystation was calculated as previously described. The number of empty oocysts present in the stock suspension was always subtracted from the number enumerated post-PEO treatment.

3:3.3 Oocyst analysis

Enumeration of oocyst excystation / spontaneous excystation

Following incubation in all experiments, each sample was centrifuged (12,500 x ***g*** for 30 sec), the supernatant aspirated and the pellet resuspended in 100 µl HBSS. Ten µl samples were then viewed under DIC and the percentage excystation was calculated as described in the maximized excystation protocol. The number of empty oocysts present in the stock suspension was always subtracted from the number enumerated post-PEO treatment.

Statistical analysis

All results are means of 2 trials, with triplicate counts of 100 – 200 oocysts undertaken per trial. The means and standard deviation of these enumerations were used in formulating graphs and tables, with a Student's 2-tailed, unpaired, t-test being used to determine significance, with a *P* value of less than or equal to 0.05 being regarded as significant. Data and statistical analysis was performed using Microsoft Excel® software.

3:4 RESULTS

3:4.1 Acidification experiments

Effect of time and temperature on the excystation of untreated C. parvum oocysts

Incubation at 37ºC generated a significant increase in spontaneous excystation ($P < 0.05$) that was time dependent when untreated oocysts were incubated in HBSS for 4 h (Table 3.1; IOWA = 6.0 ± 1.6%; MD = 8.3 ± 3.7%) or 24 h at 37°C (Table 3.1; IOWA = 24 ± 2.9%; MD = 20.9 ± 4.3%).

Table 3.1 Effect of time and temperature on the spontaneous excystation rate of *C. parvum* oocysts

Treatment	Isolate	% Excystation (0 h 37°C)	% Excystation (1 h 37°C)	% Excystation (4 h 37°C)	% Excystation (24 h 37°C)
HBSS (1 h)	IOWA	2.9 ± 1.4	2.0 ± 0.9	4.3 ± 1.3	24.6 ± 2.6
	MD	4.3 ± 2.8	7.7 ± 2.6	9.1 ± 1.5	22.3 ± 2.8
Acidified HBSS (1 h)	IOWA	0.5 ± 1.1	4.5 ± 2.0	24.4 ± 4.9	87.5 ± 3.0
	MD	0.5 ± 0.4	3.9 ± 2.1	8.0 ± 3.2	15.1 ± 2.3
HBSS (4 h)	IOWA	6.0 ± 1.6	7.3 ± 3.3	9.2 ± 3.1	22.8 ± 3.8
	MD	8.3 ± 3.7	9.8 ± 1.3	14.8 ± 2.5	20.9 ± 4.3
Acidified HBSS (4 h)	IOWA	3.9 ± 2.6	4.8 ± 2.2	16.4 ± 2.8	75.1 ± 4.4
	MD	0.5 ± 0.8	3.9 ± 1.4	6.2 ± 0.9	12.5 ± 2.0

Oocysts were incubated in the presence or absence of acidified HBSS (pH 2.75) for either 1 or 4 h at 37°C and then further incubated at 0, 1, 4 or 24 h at 37°C in HBSS. The rate of spontaneous excystation was then calculated.

Effect of purification methods on in vitro excystation

The methods used to purify the isolates had no significant effect on oocyst excystation rate when the maximised excystation protocol (Robertson, Campbell and Smith 1993b) was employed (Table 3.2, $P > 0.05$). For both isolates, excystation was greater than 94% after 4 h incubation (IOWA = 97.9 ± 1.9%; MD = 94.7 ± 1.7%) and

the indicator of infectivity, the sporozoite ratio, when assessed after 30 min was found to be very high (IOWA = 4.0 ± 0.1; MD = 3.3 ± 0.8, Table 3.2). A sporozoite ratio of 4 is the theoretical maximum. No sporozoites were detected after 4 h incubation for either isolate.

However, when the acidification step of the maximised excystation protocol was omitted (modified protocol) and the oocysts exposed to bile salts and sodium hydrocarbonate for 30 min, there was a significant increase in excystation with the MD, compared to the IOWA isolate (65.1 ± 3.4% *vs.* 2.4 ± 0.4%, Table 3.2, $P < 0.05$). The sporozoite ratio for both isolates were found to be similar to each other and both were lower than that observed in the maximised protocol, but this was not found to be significant (Modified: IOWA = 2.0 ± 1.9; MD = 2.5 ± 0.6 *vs*. Maximised: IOWA = 4.0 ± 0.1; MD = 3.3 ± 0.8, Table 3.2, $P \geq 0.05$).

At 4 h, MD isolate excystation by the modified protocol was similar to that obtained with the maximised excystation protocol (97.4 ± 1.4% *vs.* 94.7 ± 1.7%, Table 3.2). Excystation of the IOWA isolate was 39.8 ± 2.1%, significantly lower than in the maximised protocol (97.9 ± 1.9%; Table 3.2, $P < 0.01$).

Of note is the observation of sporozoites after 4 h in the modified excystation protocol. Motile sporozoites were determined in both isolates after 4 h incubation (IOWA = 3.3 ± 0.3; MD = 2.1 ± 1.4, Table 3.2) suggesting that excystation was an ongoing process in the modified protocol, whereas in the maximised protocol no sporozoites were observed at 4 h.

Effect of acidification on spontaneous excystation of C. parvum oocysts

Exposing IOWA isolate oocysts to an acidic environment (HBSS, pH 2.75) for 4 h significantly increased spontaneous excystation compared to a 1 h exposure (1 h = 0.5 ± 1.1% *vs.* 4 h = 3.9 ± 2.6%, $P < 0.01$). There was no significant difference in spontaneous excystation between the 1h and 4h incubation periods observed with the MD isolate (1 h = 0.5 ± 0.4% *vs*. 4 h = 0.5 ± 0.8%, Table 3.1).

When compared with MD, the IOWA isolate showed a significant increase in spontaneous excystation after 4 h acidification (IOWA = 3.9 ± 2.6% *vs*. MD = 0.5± 0.8%, $P < 0.05$, Table 3.1). In both isolates, treatment of oocysts with HBSS at 37°C for 1 or 4 h consistently induced significantly greater levels of spontaneous excystation than their acidified counterparts (Table 3.1, $P < 0.05$).

Incubation of oocysts in an acid environment for 1 or 4 h followed by further incubation at 37°C for up to 24 h in HBSS produced a significant increase in the rate of spontaneous excystation compared to control (HBSS treated) oocysts, but only for the IOWA isolate (Table 3.1). The MD isolate was found to have a significantly lower rate of spontaneous excystation than its control.

A time dependent increase in spontaneous excystation was observed when both isolates were exposed to HBSS for 1 or 4 h at 37°C and then further incubated for 0, 1, 4 or 24 h. This phenomenon was also observed for both isolates after initial incubation in acidified HBSS for 1 or 4 h. The IOWA isolate, however, produced the greatest increase in spontaneous excystation over time after acidification. After 1 h of acidification this isolate exhibited a low spontaneous excystation rate (0.5 ± 1.1%, Table 3.1) that increased over time with further incubation in HBSS (1 h = 4.5 ± 2.0%; 4 h = 24.4 ± 4.9; 24 h 87.5 ± 3.0, Table 3.1). A 4 h acidification had less of an impact on spontaneous excystation in the IOWA isolate, with 4 h and 24 h further incubation in HBSS causing significantly less excystation than 1 h acidification (1 h acidified HBSS + 24 h un-acidified HBSS = 87.5 ± 3.0%; 4 h acidified HBSS + 24 h un-acidified HBSS = 75.1 ± 4.4%; 1 h acidified HBSS + 4 h un-acidified HBSS = 24.4 ± 4.9%; 4 h acidified HBSS + 4 h un-acidified HBSS = 16.4 ± 2.8%, Table 3.1). This is in spite of the 4 h acidified oocysts being treated for overall longer incubation periods than the 1 h groups.

There was no significant difference in the spontaneous excystation rates observed in the MD isolate, when they were incubated for 1 h or 4 h in acidified HBSS or when they were further incubated in HBSS for 1, 4 or 24 h. These results were consistently lower for the same isolate treated with HBSS throughout the experiment (Table 3.1,

$P < 0.05$). They were also significantly lower than IOWA isolate oocysts treated with acid for 1 h or 4 h and when these were further incubated in HBSS for up to 24 h (Table 3.1, $P < 0.05$).

Table 3.2 Effect of purification methods and isolate type on the excystation of *C. parvum* oocysts

		30 min Bile	Sp. ratio	4 h Bile	Sp. ratio
Maximised Excystation Protocol	**IOWA**	86.7 ± 1.5%	4.0 ± 0.1	97.9 ± 1.9%	nd
	MD	81.5 ± 6.1%	3.3 ± 0.8	94.7 ± 1.7%	nd
Modified Excystation Protocol	**IOWA**	2.4 ± 0.4%	2.0 ± 1.9	39.8 ± 2.1%	3.3 ± 0.3
	MD	65.1 ± 3.4%	2.5 ± 0.6	97.4 ± 1.4%	2.1 ± 1.4

nd - none detected Sp. - Sporozoites

Oocysts were treated with a full, maximised excystation protocol including a 1 h acidification step (Robertson, Campbell and Smith 1993b) or excluding a 1 h acidification step (modified excystation protocol).

3:4.2 PEO treatment experiments

Control oocysts

To ensure that the oocyst suspension used throughout the study retained a consistent level of excystation, an aliquot was exposed to the maximised excystation protocol weekly and the excystation rate calculated. Over the 2 month study period, the mean percentage excystation of the stock suspensions was 96.4 ± 1.8%. The ethanol (0.6% final concentration) control neither increased nor decreased the spontaneous excystation rate of oocysts significantly ($P > 0.05$) over a 24 h incubation period at 37°C when compared to the untreated oocyst control (22.9 ± 3.8% compared with 23.6 ± 5.3%), indicating that any effect on spontaneous excystation was attributable to the PEOs.

Effect of time and temperature on spontaneous excystation in C. parvum oocysts

To determine how temperature influenced the spontaneous excystation of *C. parvum* oocysts, samples were incubated at 5ºC and 37ºC for 24 h. Incubation at 37ºC generated a significant increase in spontaneous excystation (16.0 ± 5.2%) compared

with incubation at 5ºC (-0.5 ± 1.4%, $P < 0.01$). Spontaneous excystation at 37ºC was time dependant, with a rapid increase occurring from 0 – 4 h incubation reaching a maximum of 16.0 ± 5.2% within 12 h (Table 3.3).

Table 3.3 Effect of time and temperature on *C. parvum* oocyst spontaneous excystation

Time (h)	Spontaneous excystation rate at 37ºC (%)
0	0.7 ± 0.8
4	14.4 ± 6.1
8	11.1 ± 3.9
12	16.0 ± 5.2
24	15.5 ± 4.5

Untreated oocysts were incubated for up to 24 h at 37°C in HBSS and the spontaneous excystation rate calculated at different time points. There was no significant difference between the oocyst spontaneous excystation rates after 4 h of incubation ($P < 0.05$).

Effect of PEOs on the spontaneous excystation of C. parvum oocysts

When *C. parvum* oocysts were incubated for 24 h in the presence of 0.2% PEOs, an increase in spontaneous excystation was observed, compared to untreated oocysts (Figure 3.1). Incubation in palmarosa oil produced the greatest level of spontaneous excystation (96.9 ± 1.7% of oocysts), comparable to that observed with stock oocysts exposed to the maximised excystation protocol (average over 2 months = 96.4 ± 1.8%). Oils of sweet fennel, thyme and geranium also caused a significant increase in the spontaneous excystation rate when compared to untreated control oocysts (77.1 ± 12%, 51.1 ± 9.6%, 90.9 ± 1.1%, respectively; $P < 0.01$ for all oils).

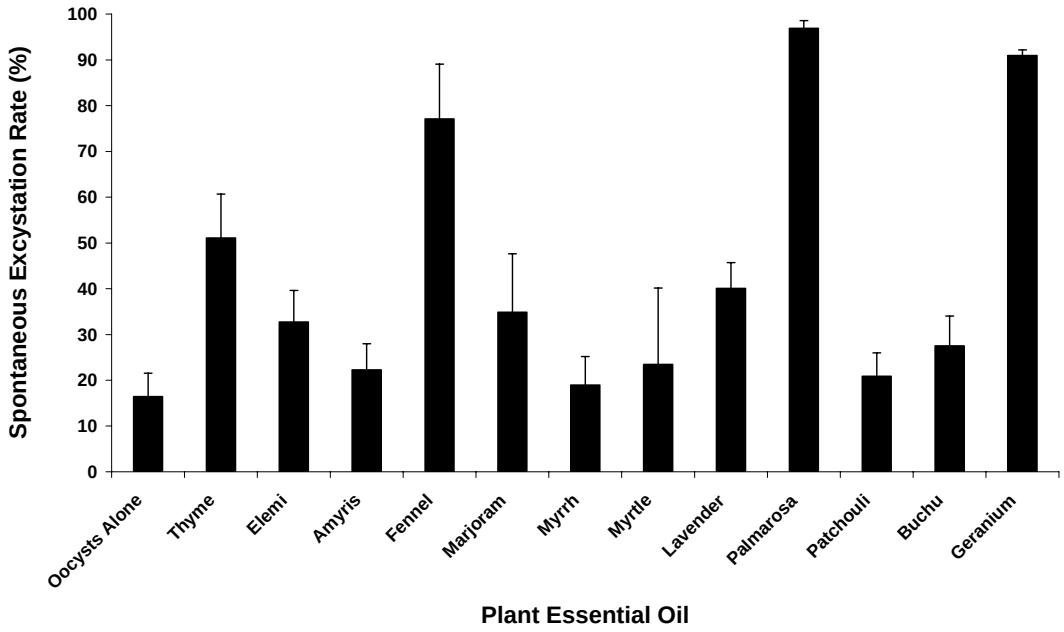


Figure 3.1 Effect of different PEOs on spontaneous excystation in *C. parvum* oocysts.
Oocysts were incubated for 24 h at 37ºC in the presence or absence of plant essential oils used at a final concentration of 0.2% v/v.

Determining whether PEO treatment influences maximised in vitro excystation when standard excystation triggers are used.

None of the PEOs tested influenced the outcome of the maximised excystation protocol following PEO pre-treatment (Appendix 7, Figure 1). This would indicate that oil treatment induces spontaneous excystation through a mechanism which does not affect the normal excystation mechanisms.

Incubation of oocysts with PEOs / constituents

PEOs found to cause high levels of spontaneous excystation of *C. parvum* oocysts *in vitro* were re-examined to determine if the action of the PEOs was due to their main constituents. In all PEOs, their main constituents caused spontaneous excystation of *C. parvum* oocysts to levels almost identical to that found with the PEO, with no significant difference being found between the effects of the whole oil and its major constituent ($P > 0.05$). After 24 h incubation, 0.16% geraniol caused 97.8 ± 1.4% spontaneous excystation with 0.2% palmarosa oil causing 96.8 ± 1.5%. This difference between the 2 excystation rates was insignificant as geraniol is the active

constituent in palmarosa oil which is responsible for the *in vitro* excystation of *C. parvum* oocysts.

Similarly, both the main constituents of sweet fennel oil (*trans*-anethole) and geranium oil (citronellol) caused spontaneous excystation of *C. parvum* oocysts at levels similar to that of their respective PEOs. After 24 h incubation, 0.14% *trans*-anethole caused 87.9 ± 2.5% spontaneous excystation with 0.2% sweet fennel oil causing 84.6 ± 5.7%. Furthermore, 0.1% citronellol caused 87.6 ± 2.7% spontaneous excystation with 0.2% geranium oil causing 91.0 ± 1.2%. These constituents can be regarded as the main active constituents in their respective oils responsible for the *in vitro* excystation of *C. parvum* oocysts.

Incubation of C. parvum oocysts with PEOs and their constituents over a 24 h time course

In order to determine the speed at which palmarosa, sweet fennel, geranium oil and their constituents induced the spontaneous excystation of *C. parvum* oocysts, samples of PEO treated oocysts were enumerated after 4, 8 12 and 24 h of incubation.

After 4 h incubation the spontaneous excystation rate of untreated control oocysts reached levels of 11.3 ± 3.8 to 18.7 ± 4.4% after which the rate of spontaneous excystation reached a plateau (Figures 3.2, 3.3 and 3.4). Both palmarosa and geranium demonstrated a time dependent increase in *C. parvum* oocyst spontaneous excystation when 0.2% of oil was incubated with the parasites (Figures 3.2 and 3.3). Both constituents demonstrated the same time dependant effect and had the same levels of activity as their respective PEOs (Figures 3.2 and 3.3, $P > 0.05$). Oils of geranium, palmarosa and their constituents did not cause a significant increase in spontaneous excystation within 4 h incubation when compared with untreated control oocysts (Figures 3.2 and 3.3, $P > 0.05$), but did demonstrate significant increases between 4 and 24 h incubation (Figures 3.2 and 3.3, $P < 0.05$).

The PEO and its main constituent which induced the most rapid increase in spontaneous excystation rate were sweet fennel and *trans*-anethole. In the initial 4 h of incubation the excystation rate increased from 0.4 ± 0.5% to 57.1 ± 2.5% for sweet fennel and from 0.4 ± 0.5% to 60.9 ± 1.1% for *trans*-anethole (Figure 3.4). The percentage spontaneous excystation remained at a plateau until 12 h and 24 h incubation, where it increased to 84.6 ± 5.7% for sweet fennel and 87.9 ± 2.5% for *trans*-anethole, a significant increase when compared to the initial 4 h incubation ($P < 0.05$).

In all cases the oil constituent performed similarly to the PEO over the time course studied, providing further evidence that they are the active constituents of the oils.

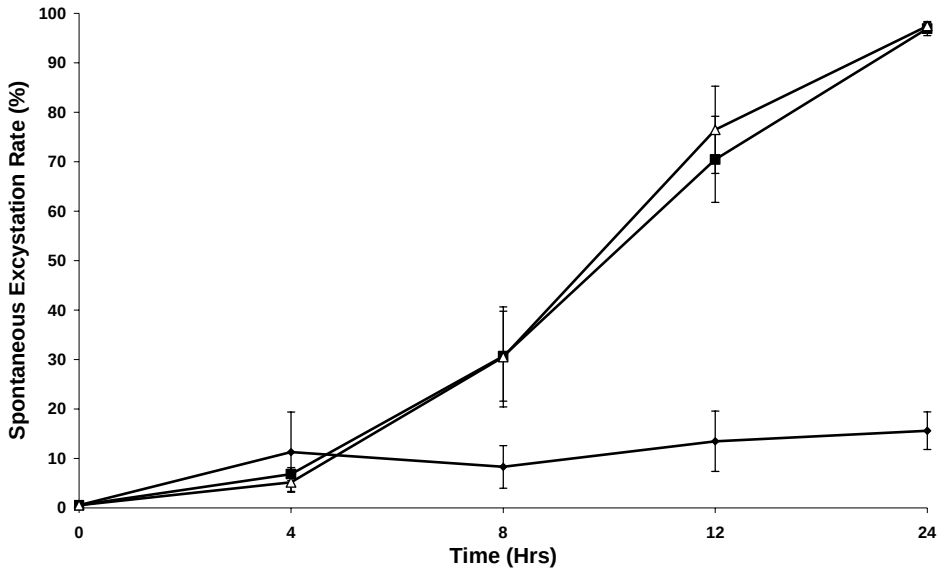


Figure 3.2 Effect of palmarosa oil and geraniol on the *in vitro* spontaneous excystation rate of *C. parvum* oocysts over time.
Oocysts were incubated for 4, 8, 12 or 24 h at 37ºC in the presence or absence of 0.2% v/v palmarosa oil (■), its main constituent, geraniol (△) at 0.16% v/v, or untreated oocysts (◆) and the rate of spontaneous excystation calculated at these time points.

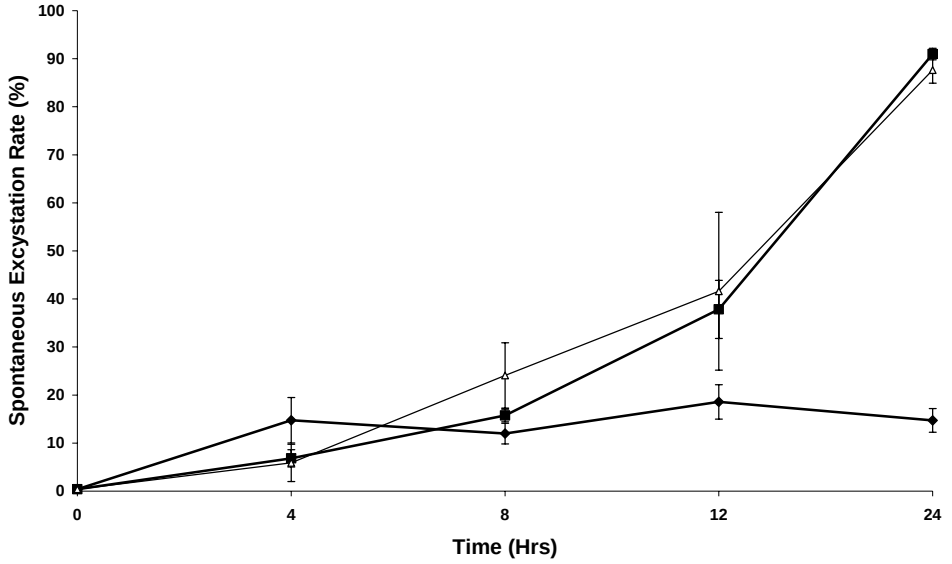


Figure 3.3 Effect of geranium oil and citronellol on the *in vitro* spontaneous excystation rate of *C. parvum* oocysts over time.
Oocysts were incubated for 4, 8, 12 or 24 h at 37ºC in the presence or absence of 0.2% v/v geranium oil (■), its main constituent, citronellol (△) at 0.1% v/v, or untreated oocysts (◆) and the rate of spontaneous excystation calculated at these time points.

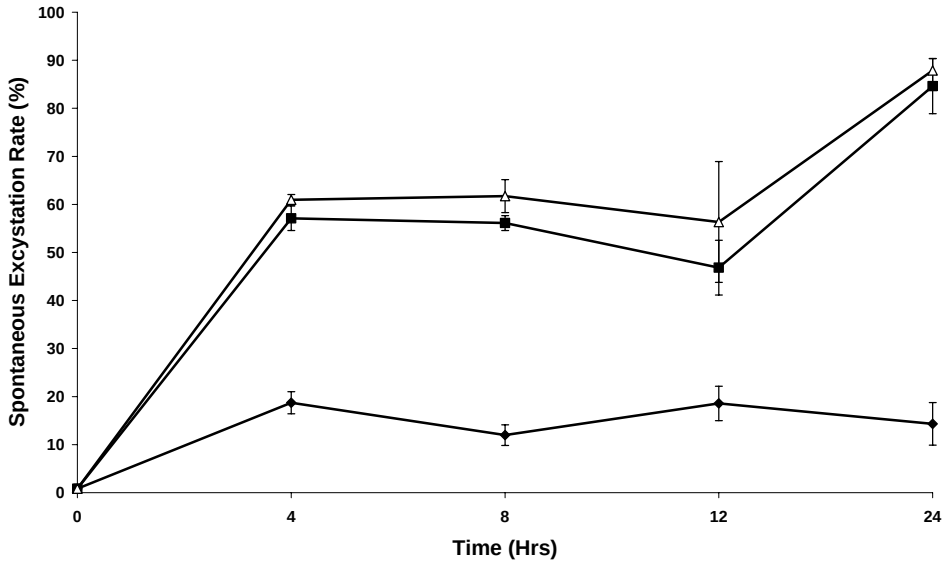


Figure 3.4 Effect of sweet fennel oil and *trans*-anethole on the *in vitro* spontaneous excystation rate of *C. parvum* oocysts over time.
Oocysts were incubated for 4, 8, 12 or 24 h at 37ºC in the presence or absence of 0.2% v/v sweet fennel oil (■), its main constituent, *trans*-anethole (△) at 0.14% v/v, or untreated oocysts (◆) and the rate of spontaneous excystation calculated at these time points.

Table 3.4 Hazard Safety Data for the most effective plant oils.

	Fennel	Anethole	Palmarosa	Geraniol	Geranium	Citronellol
Rat Oral LD_{50}	3120 mg kg^{-1}	2090 mg kg^{-1}	>5000 mg kg^{-1}	3600 mg kg^{-1}	>5000 mg kg^{-1}	3450 mg kg^{-1}
Mouse Oral LD_{50}	nd	3050 mg kg^{-1}	nd	nd	nd	nd
Guinea Pig Oral LD_{50}	No Data	2167 mg kg^{-1}	nd	nd	nd	nd
Skin Rabbit LD_{50}	>5000 mg kg^{-1}	nd	>5000 mg kg^{-1}	>5000 mg kg^{-1}	2500 mg kg^{-1}	2650 mg kg^{-1}
I.M. Mouse LD_{50}	nd	nd	nd	4000 mg kg^{-1}	nd	4000 mg kg^{-1}
S.C. Mouse LD_{50}	nd	nd	nd	1090 mg kg^{-1}	nd	880 mg kg^{-1}
I.P. Mouse LD_{50}	nd	650 mg kg^{-1}	nd	LD_{100} 1760 mg kg^{-1}	nd	nd
I.P. Rat LD_{50}	nd	900 mg kg^{-1}	nd	nd	nd	nd
I.V. Rabbit LD_{50}	nd	nd	nd	50 mg kg^{-1}	nd	nd
Skin Irritation Man	nd	nd	nd	16mg, 24H, Severe	nd	16mg, 48H, Moderate
Skin Irritation Rabbit	500mg, 24H, Moderate	nd	500mg, 24H, Moderate	100mg, 24H, Severe	500mg, 24H, Moderate	100mg, 24H, Severe
Skin Irritation Guinea Pig	nd	nd	nd	100mg, 24H, Severe	nd	100mg, 24H, Severe
Ecotoxicity Fish LC_{50}/LC_{100}	nd	nd	nd	nd	nd	nd
Ecotoxicity Crustaceae EC_{50}	nd	nd	nd	nd	nd	nd

nd = No Data available

All oils are used at 0.2% (< 2 mg ml^{-1}) in *C. parvum* experiments. In Aromatherapy oils are commonly used at concentrations of 1 – 30%. A 1000 mg kg^{-1} dose is the equivalent dose of 65g in an average 65kg adult, where 1ml of oil is roughly the equivalent of 1g. Basic safety guidelines suggest limits of between 0.5 ml (~0.5 g) and 2.5 ml day^{-1} (~2.5 g) orally (Tisserand & Balacs, 1995).

3:5 DISCUSSION

Interventions in the *Cryptosporidium* life cycle that reduce the ability of extracellular stages to infect enterocytes will reduce the number of intracellular stages and can become potentially effective treatments for cryptosporidiosis. Sporozoites are the first extracellular stages produced in the life cycle and interventions that influence their release from intact, ingested oocysts, such as premature excystation in a hostile environment or ablation of excystation can be considered as potentially therapeutic. For example, exposure of *C. parvum* sporozoites to pH 6.2 or lower causes them to lyse *in vitro* (Woodmansee *et al.* 1987). Therefore, increasing excystation in the acidic environment of the stomach can lyse sporozoites, preventing them from invading the mucosa and preventing the propagation of infection.

Conventional excystation stimuli include exposure to acid (pH ~2) followed by incubation in bile salts, reducing agents and proteases, which mimics oocyst transit through the acidic stomach to the alkaline small intestine (Smith, Nichols and Grimason 2005). However, oocysts can excyst *in vivo* and cause disease in extra intestinal locations, indicating that some host-derived triggers are not essential (Travis *et al*. 1990; French *et al*. 1995; Dunand *et al*. 1997; Smith, Nichols and Grimason 2005). Lower oocyst excystation rates at 4ºC support the hypothesis that increasing the temperature to 37ºC activates excystation, even in the absence of other host stimuli (Fayer & Leek 1984; Reduker & Speer 1985).

In this study, it was found that acidification has a major influence on the rate of spontaneous excystation, over time. Two *C. parvum* isolates from 2 different sources utilising different purification methods were used to demonstrate this observation. Increasing the temperature to 37ºC also increased *C. parvum* oocyst excystation *in vitro* over a 24 h period. The maximum spontaneous excystation levels due to temperature were 15.5 ± 4.5%, which occurred after 24 h incubation, supporting previous findings (Fayer & Leek 1984; Reduker & Speer 1985). The term ‘spontaneous’ excystation is used here to define the process of excystation *in vitro* that occurs in the absence of known triggers, apart from temperature so that it can be distinguished from excystation that occurs following maximised *in vitro* excystation

(Robertson, Campbell and Smith 1993b). Of great interest is the finding that PEOs influence the *in vitro* excystation of *C. parvum* oocysts in a temperature and time dependent manner, but in the absence of known triggers such as bile salts, reducing agents and proteases. Of the oils tested, palmarosa, geranium and sweet fennel oil were found to be the most effective at inducing spontaneous excystation of *C. parvum* oocysts *in vitro*. In these experiments both the speed at which spontaneous excystation could be induced and the constituent of the oil responsible for this effect were determined.

When 2 different isolates of *C. parvum* were incubated for up to 28 h in HBSS at 37°C there was a significant increase in spontaneous excystation but with no significant difference between the isolates. This indicates that under these particular conditions, the oocyst isolate did not influence spontaneous excystation. Both isolates showed a time dependent increase in spontaneous excystation supporting previous findings (Fayer & Leek 1984; Reduker & Speer 1985). This would also suggest that the oocyst purification methods employed by both suppliers (a modified ether extraction with discontinuous sucrose and caesium gradient for BGF – IOWA isolate; acid flocculation for MRI – MD isolate) had no effect either. Further evidence for this is provided by the similar excystation rates of both isolates when oocysts were exposed to the maximised excystation protocol of Robertson, Campbell and Smith (1993b) (IOWA = 97.9 ± 1.9% *vs.* MD = 94.7 ± 1.7%).

However, treatment of oocysts in acidified HBSS followed by incubation in acid free HBSS at 37ºC for up to 24 h affects the rate of spontaneous excystation. Comparison of the isolates showed that there was a significantly greater increase in spontaneous excystation observed for the IOWA isolate compared with the MD after acidification and then incubation for 24 h in HBSS. Incubation of oocysts in the presence of acidified HBSS for 1 or 4 h showed a slight, but significant increase in the rate of spontaneous excystation for the IOWA isolate with the 4 h acidification but not for the MD isolate (Table 3.1, $P < 0.05$). The latter isolate failed to show any difference in spontaneous excystation between the 2 incubation periods.

When these acid treated oocysts were then exposed to HBSS for 24 h, the IOWA isolate had a spontaneous excystation rate of 87.5 ± 2.5% and the MD isolate spontaneous excystation rate of 15.1 ± 2.3%. Not only was this rate significantly lower than the IOWA isolate, it was also significantly lower than HBSS treated controls for the MD isolate over the same incubation period (acidified MD = 15.1 ± 2.3% *vs*. control MD = 24.6 ± 2.6%, Table 3.1, $P < 0.01$). This reduction in spontaneous excystation observed for the MD isolate may have been influenced by the increased exposure of these oocysts to low pH. Prior to experimental exposure, the MD isolate is exposed to acid (0.01% v/v concentrated HCl) during the MRI purification procedures whereas the IOWA isolate is exposed to acid only during the experiments.

This acid sensitivity of oocysts was further investigated by employing the maximised excystation protocol of Robertson, Campbell and Smith (1993b) and a modification of this protocol where the acidification step was omitted, i.e. exposing the oocysts to other known triggers of excystation such as temperature and bile salts. Experiments showed that the MD isolate exposed to the modified protocol achieved excystation rates similar to that demonstrated with the maximised protocol (maximised = 94.7 ± 1.7% *vs*. modified = 97.4 ± 1.4%, Table 3.2). However, this was not observed with the IOWA isolate. With IOWA, acidification was a required step in the excystation protocol and its omission caused a significant reduction to the excystation rate (maximised = 97.9 ± 1.9% *vs*. modified = 39.8 ± 2.1%, $P < 0.01$). Comparison of both isolates treated with the maximised protocol showed no significant differences for excystation rates after 30 min and 4 h and no significant difference between their sporozoite ratios measured after 30 min.

The MD isolate, as previously mentioned, had already been exposed to acid in its purification and when exposed to the modified excystation protocol the oocysts began excysting at an accelerated rate compared to the IOWA isolate (MD 30 min = 65.1 ± 3.4% *vs*. IOWA 30 min = 2.4 ± 0.4%, Table 3.2, $P < 0.01$). When a maximised protocol was used, the IOWA and MD isolates showed similar

excystation rates at 30 min and were significantly higher than that observed for the modified protocol (MD = 81.5 ± 6.1%; IOWA = 86.7 ± 1.5%, Table 3.2, $P < 0.05$).

Acidification appears to be a requirement for maximising *C. parvum* oocyst excystation. However, the isolate and its method of purification bear an important role in excystation. Purification of oocysts using an acidic method appears to desensitise the oocysts to spontaneous excystation following subsequent acid exposure. Oocysts which have been purified without the use of acid are sensitive to the action of acid, which will enhance their spontaneous excystation and also their excystation induced by the maximised protocol. The absence of acid inhibits excystation induced by the modified protocol.

Oocysts of the IOWA isolate were used in PEO treatment experiments to investigate their activity on oocysts not previously primed for excystation by acidification. Oocysts incubated in the presence or absence of sweet fennel or palmarosa oil at 5ºC do not excyst spontaneously (Appendix 7, Figure 2). Therefore, temperature is a major factor in this phenomenon. Of the 12 PEOs tested, 4 significantly affected the spontaneous excystation of *C. parvum* oocysts ($P < 0.01$, Figure 3.1). Those PEOs causing spontaneous excystation at 37ºC, significantly above untreated control levels, included palmarosa, sweet fennel, thyme and geranium oils (96.9 ± 1.7%, 77.1 ± 12%, 51.1 ± 9.6%, 90.9 ± 1.2%, respectively). This is particularly the case with palmarosa oil, which generated excystation rates similar to that found with oocysts exposed to the maximised *in vitro* excystation method of Robertson, Campbell and Smith (1993b) (96.9 ± 1.7% compared with 96.4 ± 1.8%). To the author's knowledge, this is the first time that the effect of PEOs on spontaneous excystation of *C. parvum* has been reported.

Incubating oocysts at 37ºC in the presence of PEOs increased spontaneous excystation significantly compared to control values (e.g. from ~19% to ~96% for palmarosa oil, $P < 0.01$). Therefore, the triggers for excystation must be different. The act of spontaneous excystation in the absence of all recognised triggers except temperature, would suggest that the PEOs might be acting in 1 of 2 ways i) that the

PEOs augment the effect of temperature on oocyst excystation by increasing oocyst wall permeability or ii) the reverse hypothesis, that an increase in temperature causes an activation of spontaneous excystation initiated by PEOs, especially with oils of palmarosa, geranium, sweet fennel and thyme, respectively.

Oocysts which did not spontaneously excyst with the application of PEOs were found to do so in the presence of the classical triggers of the maximised excystation *in vitro* protocol, suggesting that pre-incubation with PEOs doesn't inhibit the actions of *in vitro* excystation triggers.

A potential use of PEOs may be as a means of limiting the propagation of infection of *C. parvum* in neonatal livestock, perhaps by including the PEO in their feed, providing the PEO can survive passage through the gut to the site of infection. PEOs can be safely kept for periods up to 2 years with no changes to its composition or its constituents by oxidation providing they are stored in the dark, in airtight containers and at 2 – 5°C. Oxidation can occur much more quickly if PEOs are not kept in these conditions and the decomposition products may be toxic.

Oils of thyme, sweet fennel, palmarosa and geranium are all readily available from a variety of sources and are currently used in the food and scent industries. Hazard safety data for these oils is limited (Table 3.4). However, due to their extensive use in various applications they are 'generally regarded as safe' (GRAS) by the United States' FDA.

Initially, the composition of palmarosa, geranium and sweet fennel oil were determined by GC-MS means with the assistance of F.D. Copeland & Sons. From this it could be determined that the monoterpene alcohol, geraniol, comprised 80% of palmarosa oil, the aromatic ether, *trans*-anethole comprised 68% of sweet fennel oil and that the monoterpene, citronellol comprised 39% of geranium oil. Incubation of these constituents on their own with oocysts at concentrations equivalent to that found in the PEO were able to elicit the spontaneous excystation of *C. parvum* oocysts *in vitro* at levels commensurate with their respective PEOs (sweet fennel =

84.6 ± 5.7% *vs*. *trans*-anethole = 87.9 ± 2.5%; geranium = 91.0 ± 1.2% *vs*. citronellol = 87.6 ± 2.7%; palmarosa = 96.8 ± 1.5% *vs*. geraniol = 97.8 ± 1.4%). These results would indicate that the constituents investigated were responsible for the actions of the PEO.

The almost complete excystation of oocysts caused by geraniol and palmarosa oil at levels comparable with untreated controls undergoing the maximised excystation protocol may have beneficial applications. Whilst it takes 24 h of exposure to elicit almost 100% spontaneous excystation, there may be potential for the use of PEOs, or their constituents, in the treatment of farmyard faecal slurry so that the risk of viable oocysts being present in any water contaminated by such material can be reduced. By inducing excystation of oocysts outwith the body, the released sporozoites are exposed to conditions that are hazardous to their survival. The purpose of the oocyst wall is to provide a safe and protected environment for the sporozoites to exist until they have reached conditions which are optimal for their survival and invasion of enterocytes.

Both palmarosa and its main constituent, geraniol, appear to be the most effective method of causing excystation over a 24h period. This constituent also has the advantage of being tested extensively for hazard data and is known to be bio-degradable in the soil (United States' Environmental Protection Agency (EPA 2004)). Geraniol is also the metabolite of geranyl-acetate. It is metabolises in the intestines of mammals when it is consumed. Where geraniol is required, it may be possible to administer geranyl-acetate and this takes advantage of the mammal metabolic processes to produce geraniol *in situ*. Geranyl-acetate is also commonly used in foods as flavouring and has also been given GRAS status by the FDA.

The speed at which spontaneous excystation occurred was also examined. A time dependant increase in spontaneous excystation was observed for each PEO and their constituents. Both palmarosa / geraniol and geranium / citronellol were able to exert their maximal effect 12 – 24 h after incubation, but their 4 h action was limited. Sweet fennel and *trans*-anethole, however, exerted between 57.1 – 60.9%

spontaneous excystation within 4 h. While this level of excystation is lower than the overall rate after 24 h for these compounds (84.6 and 87.5% respectively), it is significantly higher than that obtained with palmarosa / geraniol and geranium / citronellol over the same 4 h (palmarosa / geraniol = 5.2 – 6.8%; geranium / citronellol = 5.9 – 6.8%). It is also significantly higher than untreated oocysts after 4 h incubation at 37°C (Figure 3.4, $P < 0.05$). Considering that passage through stomach can take up to 4 h, this is an intriguing result. Potentially, there may be applications for sweet fennel oil or *trans*-anethole as a means of excysting *C. parvum* oocysts prior to entry into the small intestine.

The maximised *in vitro* excystation method of Robertson, Campbell and Smith (1993b), is completed within 5 h (pre-acidification of oocysts for 1 h, followed by incubation in bile salts for 4 h). While the spontaneous excystation rate induced by sweet fennel oil or *trans*-anethole is significantly lower than the maximised excystation rate at the same time point, it is still an interesting result. This oil and its constituent are working in a manner independent of acid and bile to induce an initial high rate of excystation. This may be due to the PEO and constituent causing the oocyst wall to become more permeable, as would normally happen during acidification or perhaps augmenting temperature induced permeability.

It is well recognised that if excystation of *C. parvum* oocysts occurs in the stomach, which is too acidic for sporozoite survival (optimal pH 6.2, Woodmansee *et al.* 1987), sporozoites lyse, which reduces both enterocyte invasion and the subsequent propagation of infection. By utilising the speed at which *trans*-anethole and sweet fennel oil are able to cause spontaneous excystation, it may be possible to induce this action at a site which is unfavourable to the sporozoite survival, e.g. the stomach.

Further, the slower acting geranium, palmarosa, citronellol and geraniol may also have chemotherapeutic uses for cryptosporidiosis. By inducing the release of sporozoites prior to the release of oocysts in faeces, we can limit the propagation of infection, not just in humans as an immediate treatment, but also prophylactically in cattle and other livestock. This is a major source of infection for humans (Caccio *et*

al. 2005). Providing that the pharmacokinetics of these constituents are found to be favourable for a sustained exposure in the gut, they may become prime novel drug candidates. Considering that infected humans may shed oocysts for at least 2 weeks in 82% of infected people, 3 weeks in 42% and 4 weeks in 21% (Baxby, Hart and Blundell 1985), the slower action of geranium and palmarosa oils may be used to induce excystation of oocysts prior to or just after excretion, again limiting the propagation of infection.

Some hazard information is currently available for the compounds citronellol, geraniol and *trans*-anethole as well as the essential oils geranium, palmarosa and sweet fennel. All 3 constituents and PEOs are currently used in food as flavourings with the FDA considering them all to be GRAS for food use (FDA Food additive status list 2006). This may allow their potential use internally as chemotherapeutic compounds. Safety information regarding these constituents and PEOs is given in Table 3.4.

The World Health Organisation (WHO) states that citronellol most likely metabolises to harmless substances and that the majority of the compound is eliminated in the urine. This is based on data on a corresponding substance, geraniol. It is also an indication that these 2 compounds may share common pharmacokinetics. Indeed, geraniol, a naturally occurring straight chain terpene alcohol, is structurally related to citronellol and differs only by the presence of an additional double bond (Cantwell *et al*. 1978). Geraniol is produced by many flowering plants, including geraniums and citrus fruits, in the scent glands of honey bees and is found in over 250 essential oils. It is used commercially for its fragrance, being used in the manufacture of detergents, soaps, creams, lotions, cosmetics and aromatherapy products (Höschle & Jendrossek 2005). This chemical is also used as a synthetic flavouring agent in beverages, ice cream and sweets and is granted GRAS status under section 409 of the Federal Food, Drug and Cosmetic Act (FFDCA 2004). Both geraniol and citronellol do not present any known health risks to humans (Höschle & Jendrossek 2005).

Trans-anethole is used as a flavouring substance in foods at approximate levels ranging from 2.5 ppm in gravies to 1500 ppm in chewing gum (Newberne *et al*. 1999). This roughly equates to concentrations from 0.00025% (2.5 μg g^{-1}) to 0.15% (1500 μg g^{-1}). In mice, rats and humans, orally administered *trans*-anethole and other alkoxypropenylbenzene derivatives are rapidly absorbed from the gastro-intestinal tract and they are completely metabolized mainly in the liver to yield polar acidic metabolites, which are conjugated and eliminated primarily in urine (Fritsch, De Saint Blanquat and Derache 1975; Le Bourhis 1968 and 1970; Solheim & Scheline 1973 and 1976).

Other than food or fragrance uses, both geraniol and citronellol are being currently investigated for potential chemotherapeutic use. Research has demonstrated that geraniol is an effective insect repellent when tested against the mosquitoes *Aedes albopictus* (associated with the transmission of dengue, eastern equine encephalitis and dog heartworm and potentially with St. Louis and LaCrosse encephalitis viruses)*, Culex nigripalpus* (a possible vector of West Nile virus) *and Ochlerotatus triseriatus* (vector for LaCrosse encephalitis viruses) (Barnard & Xue 2004). Citronellol is already utilised in many insect repellents. Both geraniol and palmarosa oil have antimicrobial properties (Prashar *et al*. 2003; Duarte *et al*. 2007), both geranium oil and citronellol have antifungal properties (Shin & Lim 2004) and *trans*-anethole and sweet fennel are antimicrobial acting on a range of bacteria, yeasts and fungi (De *et al.* 2002; Fyfe, Armstrong and Stewart 1997).

It is clear that these three PEOs and their constituents have a role to play in the excystation of *C. parvum* oocysts *in vitro*. The only other triggers required for spontaneous excysation to occur in the presence of these constituents appears to be an increase in temperature and time. The mechanism by which sweet fennel, palmarosa, geranium, *trans*-anethole, geraniol and citronellol are able to induce this form of spontaneous excystation is still unclear. It is generally agreed that *in vivo* requirements for excystation are an increase in temperature, acidification prior to exposure of bile salts, reducing agents and proteases found in the small intestine, although what role each has to play is still being investigated (Smith, Nichols and

Grimason 2005). It is known that both an increase in temperature and acidification will increase the permeability of the oocyst wall. The tranduction of signals through an oocyst wall made increasingly permeable by elevated temperature have been shown to elicit responses in the sporozoites which can initiate excystation (Forney *et al*. 1996a and 1996b; Okhuysen *et al*. 1996; Smith & Ronald 2002).

The increased permeability by temperature has previously been shown to allow the ingress of small molecules such as the nuclear fluorogenic dye 4′6-diamidino-2-phenyl indole (DAPI) in a time dependent manner. This molecule has a formula weight (FW) of 457.48 and with increased temperature and time, it may migrate though the oocyst wall, although a further requirement *in vitro* is acidification. This permits a more rapid entry of the dye through an increased permeability of the wall (Campbell, Robertson and Smith 1992). The FWs of the terpenes and aromatic ether investigated are less than half that of DAPI (geraniol FW 154.25; *trans*-anethole FW 148.2; citronellol FW 156.27), which would indicate that they may be able to pass through a less permeable wall, or at least one which would preclude the passage of DAPI. The lipophilic properties of PEOs and the presence of lipids in the oocyst wall (Smith & Ronald 2002) may help augment this permeability. This might be through being an alternative to acidifcation for increasing permeability and allowing the easier entry of constituents of the oil into the oocyst. This may potentially affect the sporozoites and initiate excystation. What is not known is if this possible increase in wall permeability enhanced by PEOs is selective (i.e. allowing controlled excystation) or non-selective, where ‘pores’ are formed, rapidly changing the permeability of the oocyst and exposing the sporozoites to the PEOs which may damage them, thus preventing their invasion of enterocytes.

CHAPTER 4

Trypanosoma cruzi

The activity of various PEOs and their constituents, on *Trypanosoma cruzi* epimastigote viability *in vitro*.

4:1 ABSTRACT

The trypanocidal action of the PEOs sweet fennel, lavender, amyris, marjoram, palmarosa, patchouli, myrrh, myrtle, elemi, thyme, buchu, geranium and some of their constituents (α-pinene and eucalyptol) was investigated on *T. cruzi* epimastigotes *in vitro*. The MIC of the oils and constituents against *T. cruzi* was determined by cell counting in a Neubauer chamber. Treatment with oils and their constituents demonstrated that they reduce parasite viability, with myrtle and elemi essential oil being the most active (MICs for both oils = 0.00125% [12.5 μg ml^{-1}]). Comparison of IC_{50} values with that cited in the literature for benznidazole demonstrated that both myrtle and elemi oils had activity that was within the range of benznidazole (myrtle IC_{50} = 2 μg ml^{-1}; elemi IC_{50} = 3 μg ml^{-1}; benznidazole IC_{50} = 0.73 – 4.4 μg ml^{-1}). All of the PEOs investigated were able to reduce parasite viability within 2 h when used at their MICs. The results presented here demonstrate those plant oil/s which could have a role to play in the treatment of Chagas' disease.

4:2 INTRODUCTION

The disease caused by *T. cruzi*, Chagas' disease, is named after its discoverer, Carlos Chagas and occurs in the Americas, ranging from the Southern United States of America, through Mexico and into mainly rural areas of Central South America and as far South as Argentina (CDC Chagas' disease fact sheet, accessed 28th August 2008). The increased distribution of Chagas' disease in rural areas compared with urban and suburban population centres can be accounted for by the types of dwellings used by the human population. As the Reduviid bugs' natural habitat is in shrubs and bushes, the thatched roofs in rural dwellings make a good substitute for its natural habitat with the added bonus of easy access to blood meals. With the loss of natural habitat and the use of more 'modern' building materials of concrete, bricks and slate roofs in urban / suburban areas, there are fewer habitats in which the Reduviid bug may survive, thus reducing the interaction of Reduviid bugs with humans and so reducing the distribution of Chagas' disease in these areas.

Chagas' disease is of major medical importance throughout Latin America due to its public health and economic impact. Approximately 16 – 18 million people are infected, with up to 120 million at risk of infection (25% of the population of Latin America). It is estimated that 25 – 30% of those infected suffer chronic irreversible damage to the heart and digestive tract leading to approximately 50,000 fatalities being reported every year (WHO Technical Report Series 2002). More than 10 million people carry the protozoan parasite *T. cruzi*, which multiplies inside cells, particularly those of the heart and smooth muscle (Miles 1997). Infection in humans leads to varied clinical presentations with mild symptoms. Patent parasitaemia lasting 40 – 60 days can occur, with the disease often not being recognized at this stage. In the chronic phase of infection, up to 30% of infected people may develop severe abnormalities on the electrocardiogram and present with chagasic cardiomyopathy (WHO 2002).

Chagas' disease is especially dangerous at the present time as there are no satisfactory prophylactic or curative agents available for the chronic stage of the infection. Once the disease is contracted by a patient, the individual remains infected

until death. Although there are drugs (nifurtimox and benznidazole) available for treatment of the acute stage of infection, symptoms are rarely recognised in time for chemotherapeutic intervention to take place.

The acute phase of infection can be characterised by an intense and localised inflammatory response at the site of inoculation, forming a small red nodule/lesion (chagoma). Where infection occurs through the conjunctiva of the eye, inflammatory responses cause oedema of the conjunctiva and eyelid with a swelling of the preauricular lymph node. These ocular symptoms collectively constitute the classical sign of acute infection known as Romaña's sign. The acute phase is often asymptomatic and whilst there are effective drug treatments (nifurtimox and benznidazole) for this stage, it is often difficult to diagnose. Patient compliance with treatment regimens is often poor, as the treatment is lengthy.

Resistance to these drugs are now being reported (Buckner *et al.* 1998) and both drugs require a high degree of patient compliance for optimum results. Nifurtimox is given at a dose of 5 mg/kg/day orally, slowly increased to 15 mg/kg/day (divided over 3 doses) for 2 to 4 months during the acute cycle of infection. However, this drug is now no longer being produced and whilst there are still stocks available, the cost to the patient can be prohibitive ($48 US per treatment regimen, the equivalent of one month's pay for a Bolivian miner; Weir 2006). Benznidazole is generally given at 5 – 10 mg/kg/day orally in 2 daily doses for 1 – 2 months. Common side effects for both drugs include neurotoxicity, anorexia, nausea and leukopoenia. Nifurtimox exhibits these the most frequently whilst benznidazole shows a greater degree of skin manifestations such as rashes in as much as 30% of patients (Castro, de Mecca and Bartel 2006). Benznidazole is no longer produced by the original manufacturer (Roche) and is now produced by the Brazilian Government who were given the commercial rights and equipment to manufacture this drug. Problems with the supply of the primary constituent of this drug have led to delays in its distribution, often up to 4 months (Weir 2006).

It is clear that alternative therapuetics must be sought and one particular source of novel drugs may be from the plant world. Plants and their extracts have been used for many centuries as treatments for ailments, from headaches to parasite infections. However, it is only in the past 20 – 30 years that scientists have seriously begun to determine whether plant-derived traditional remedies are effective and if so, their mode of action (Anthony, Fyfe and Smith 2005). In the last 17 years, 199 papers have been published concerning the antitrypanosomal activities of plants and their identified constituents, many of which have come through the investigation of traditional native remedies (PubMed query accessed 10th January 2008). Recently Vieira *et al*. (2008) published an investigation into 215 plants from Northeastern Brasil for their activity against *T. cruzi* epimastigotes *in vitro*. Of these plants only 4 were found to have significant trypanocidal efficacy with a combination of 8 different constituents from these plants being found to be active. Flavenoids from plants have been investigated with some success (Sülsen *et al*. 2007), as have polyacetylenes (Senn *et al*. 2007) and terpenic compounds (Mikus *et al*. 2000; Hoet *et al*. 2006; Santoro *et al*. 2007a).

Axenic culture of *T. cruzi* epimastigotes/metacyclic trypomastigotes has been possible for many years (Contreras *et al*. 1985). Axenic culture is commonly used for the screening of compounds for novel antitrypanosomal effects as well as studies on the biology of *Trypanosoma* parasites (Bringaud, Rivière & Coustou 2006; Schinella *et al*. 2002). Epimastigotes/metacyclic trypomastigotes of *T. cruzi* are deposited on the skin in the faeces and urine from the Reduviid bug as it takes a blood meal from its host (Chapter 1, pp 43 – 44; Figure 1.9). When the bite wound is scratched, epimastigotes enter the bloodstream of the host and rapidly invade host cells whereupon they differentiate into replicative amastigotes. The prevention of infective epimastigotes/metacyclic trypomastigotes entering the host cells is an advantageous step in limiting the propagation of infection. As the epimastigotes can be readily axenically cultured and are found on the skin, PEOs may be of use in this initial treatment of infection by ridding the skin of infective epimastigotes. The complete life cycle of *T. cruzi* is given in Chapter 1.

The method most commonly used to screen drugs for the treatment of Chagas' disease (microscopic counting of viable trypanosomes), can be very accurate but it is time-consuming, labour-intensive and dependent on the observer. Another method which may alleviate some of these problems is the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. This is comparatively quick and accurate, but requires careful attention to experimental design and interpretation of the results (Muelas-Serrano, Nogal-Ruiz and Gómez-Barrio 2000). A problem with this assay is that the reduction of the tetrazolium salt to a formazan product requires further solublisation in the medium before recording absorbance readings. This further complicates the assay, as it requires several procedures and manipulations of the test organism before complete analysis is carried out. This can lead to error in the final analysis. A less complicated method has been developed by the company Promega, where a novel tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) has been developed in a modification of the MTT assay. The MTS assay is simple, requiring the addition of a set dilution of reagent to the well of a 96 well microtitre plate containing a volume of cells or parasites. The biochemical reduction of the tetrazolium salt occurs with live organisms and the formazan product formed is soluble in tissue culture medium. The quantity of formazan product measured by absorbance at 492nm is directly proportional to the number of living cells in culture (Promega Technical Bulletin; http://www.promega.com/tbs/tb245/tb245.html accessed 8th January 2008).

There is an intense search for new trypanocidal drugs, with plant extracts and PEOs appearing to be promising alternatives to classical chemotherapeutics. Thus the purpose of this series of experiments is several fold: (i) to establish the working concentrations of *T. cruzi* epimastigotes in a 96 well microtitre assay system, (ii) to determine whether the diluent (ethanol) can be safely used without affecting epimastigote viability (iii) to examine the use of a biochemical, colourmetric assay (MTS assay) or morphological analysis with the addition of vital dye (Trypan blue, motility and morphological assay [TBMM]) to accurately determine viability, so that statements could be made as to the efficacy of any tested plant essential oils, (iv) to

analyse the activity of 12 different PEOs on the viability of *T. cruzi* epimastigotes *in vitro* and (v) to determine their active constituents and potentially their mode of action.

4:3 MATERIALS AND METHODS

4:3.1 T. cruzi in vitro culture

Source and maintenance of T. cruzi epimastigotes

Epimastigotes of *T. cruzi* (Silvio X10 isolate) were maintained axenically in sealed flat sided 110 mm x 16 mm culture tubes (Nunc, Denmark) at 25ºC, in Optimem medium (Gibco-BRL, Paisley, UK) supplemented with 10% (v/v) heat-inactivated foetal bovine serum (complete medium) (Gibco-BRL) and filled to ~50% capacity. Sub-culturing was performed routinely at 7 day intervals by harvesting from the culture tubes and then pipetting 1 ml of the suspended epimastigotes into ~6 ml of fresh complete medium.

T. cruzi enumeration for experiments

Epimastigote enumeration was performed using an improved Neubauer haemocytometer, with epimastigote viability determined by Trypan blue inclusion / exclusion where dead cells (stained) were determined microscopically. A lack of motility and changes to the morphology served as corroborating parameters of cell death (TBMM). Briefly, epimastigotes were harvested from the culture tubes by centrifuging the culture tubes (500 x ***g***, 5 min). The supernatant was aspirated to waste and the pellet resuspended in 1 ml complete medium. A 20 µl aliquot of this suspension was added to 80 µl of complete medium in a 1.5 ml microcentrifuge tube to dilute the numbers of epimastigotes for easier counting. A 20 µl aliquot of this suspension was then added to 20 µl 0.4% w/v Trypan blue solution (Gibco-BRL) and mixed thoroughly. After mixing, 10 µl were dispensed into both chambers of an improved Neubauer haemocytometer, allowed to settle (30 – 60 sec) and then enumerated. Epimastigote enumeration was determined under bright field microscopy in the manner previously described in Chapter 2 for *G. duodenalis* trophozoites.

Comparison of incubation at 25°C and 37°C in the MTS assay and the optimisation of T. cruzi epimastigote concentrations

Epimastigotes at concentrations from 5 x 10^3 – 1 x 10^7 epimastigotes ml^{-1} were incubated over 24 h at 25°C using 100 µl $well^{-1}$ in 2 x 96 well microtitre plates. Following incubation, 20 µl of MTS reagent was added to each well containing 100

µl of parasites. One 96 well plate was then further incubated at 25°C with the other at 37°C in the dark. After 1.5, 3, 4 and 6 h for 25°C and 1, 2, 3, 4 h for 37°C incubation, the absorbance at 492nm for each well was performed. From these results a time of 4 h at 25°C or 3 h at 37°C was demonstrated to be optimal for viability assessment using MTS with a concentration of 1 x 10^7 epimastigotes ml^{-1}.

4:3.2 Experimental treatments of epimastigotes

Viability in ethanol

It had been previously demonstrated that ethanol could be used as a diluent without affecting *G. duodenalis* viability (Chapter 2). To determine whether this diluent could have any affect on *T. cruzi* epimastigotes, a final concentration equivalent to that obtained with a diluted PEO was assessed. A volume of 16 µl 75% v/v EtOH stock was diluted in 1 ml of complete medium, with vortexing for 20 sec and 50 µl of this being immediately added to 50µl of 2 x 10^7 epimastigotes ml^{-1}. Incubation occurred in sealed 96 well microtitre plates at 25°C for 24 h following which parasite viability was assessed by microscopy and Trypan blue inclusion/exclusion as well as the MTS assay.

PEO preparation

All PEOs were diluted in absolute ethanol to provide 25% v/v stock solutions (25% PEO, 75% absolute ethanol), which were used in these experiments. The full list of PEOs investigated is given in Appendix 1.

Incubation of T. cruzi epimastigotes with PEOs

After 5 days of culture, epimastigotes were harvested. Experiments were performed in sterile, 96 well microtitre plates covered with plate sealer film and lids (Costar, Corning). Final concentrations of 0.02 % of PEOs were initially used. A final concentration of 0.02% in the microtitre well was achieved by diluting 16 µl of PEO (25% v/v stock) in 1 ml of complete medium and vortexing for 20 sec with 100 µl of this being immediately added to 100µl of 1 x 10^7 epimastigotes ml^{-1}.Where 100% killing of epimastigotes was observed, the active PEOs were titrated using doubling dilutions starting at 0.02% to determine their MICs. The microtitre plates were sealed

and incubated for 24 h at 25ºC, after which the epimastigotes were enumerated. After 2 h of incubation the parasites were examined microscopically in their wells and also again at 24 h prior to enumeration of viability.

Controls consisted of 1 x 10^7 epimastigotes ml^{-1} in 200 µl complete medium in the absence of PEOs. Unfortunately, the reference drugs for the treatment of American trypanosomiasis were unavailable and a drug control was not able to be used. A control with epimastigotes exposed to 56°C for 10 min was used as a visual comparison. All samples were prepared in triplicate and each experiment in duplicate.

Incubation of T. cruzi epimastigotes with PEOs and PEO constituents over a 2 h time course

During the 2 h observation period it was noted that there was evidence of antiparasitical activity. A 2 h time course experiment was then employed to determine how quickly PEOs exhibited antitrypanosomal activity. *T. cruzi* epimastigotes were incubated in sealed microtitre plates for 2 h in the presence or absence of PEOs at their MIC (Table 4.1).

Table 4.1 Minimal inhibitory concentration of PEOs used in experiments.

MIC (%)	PEO
0.01	Buchu
0.01	Thyme
0.005	Lavender
0.005	Palmarosa
0.0025	Amyris
0.0025	Geranium
0.0025	Marjoram
0.0025	Myrrh
0.0025	Patchouli
0.0025	Sweet Fennel
0.00125	Elemi
0.00125	Myrtle

Incubation of T. cruzi epimastigotes with PEO constituents

Continuing from the PEO titration experiments, one particular PEO exhibited the greatest antitrypanosomal properties, as assessed by MIC when compared with the other 11 PEOs. Oil of myrtle was examined for its composition using GC-MS as previously described in Chapter 2 with the composition of the PEO given in Appendix 3. The major constituents of myrtle oil were the monoterpenes, α-pinene and eucalyptol (syn. 1,8-cineole) comprising 51.72% and 22.25% of the PEO respectively. These constituents were prepared in the same manner as their PEOs and used in experiments.

The constituents α-pinene and eucalyptol were incubated for 24 h with the parasites initially at the equivalent myrtle oil concentration of 0.02% (i.e. α-pinene = 0.01% and eucalyptol = 0.0044%). A titration of doubling dilutions was performed so that final concentrations of α-pinene were 0.01, 0.005, 0.0025 and 0.00125% and eucalyptol at 0.0044, 0.0022, 0.0011 and 0.00055%. These constituents were also incubated in combination, in the presence of epimastigotes at the above concentrations so that 0.01% α-pinene was added with 0.0044% eucalyptol, 0.005% α-pinene with 0.0022% eucalyptol and so on.

4:3.3 Epimastigote culture analysis

T. cruzi cell count assay

Epimastigote enumeration was performed using an improved Neubauer haemocytometer and epimastigote viability determined by TBMM. Epimastigotes were harvested from wells, the contents of triplicate wells were combined into 1.5 ml microcentrifuge tubes and centrifuged (10 sec, bench top centrifuge 5410, Eppendorf GmbH, Germany). The supernatant was aspirated to waste and the pellet resuspended in 100 µl complete medium and 100 µl 0.4% w/v Trypan blue solution (Gibco, UK) and mixed thoroughly. After mixing, 10 µl were dispensed into both chambers of an improved Neubauer haemocytometer, allowed to settle (30 – 60 sec) then assessed as previously described in Chapter 2.

Epimastigote viability was also determined in experiments using the CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (Promega, USA; MTS Assay) which uses the novel tetrazolium compound, MTS and the electron coupling reagent, phenazine ethosulphate (PES). The procedures followed were as previously described in section 4:3.1 of this chapter.

The recommended medium to use for the MTS assay is RPMI 1640 although any medium which does not absorb light at 492nm may be used. As the parasites were cultured in Optimem, this was used in the assay. In all experiments, wells containing Optimem were used as 'blanks' to assess its absorption of light at 492nm and were subtracted from all absorption readings. At the determined incubation time and temperature of 4h and 25°C for use in experiments (Section 4:3.1) Optimem has a mean absorbance of 0.342 ± standard deviation x3 of 0.044. This would mean that for epimastigotes to be determined as being viable by formazan reduction, the lowest uncorrected absorbance would have to be greater than the highest positive standard deviation for Optimem, i.e. > 0.386.

Statistical analysis

In this series of experiments epimastigotes of *T. cruzi* from each microtitre well from 2 experiments were enumerated, providing a total of 6 enumerations for each PEO concentration tested. The means and standard deviation of these enumerations were used in formulating graphs and tables, with a Student's 2-tailed, unpaired, t-test used to determine significance with a *P* value of less than or equal to 0.05 being regarded as significant. The construction of dose–response curves and calculation of IC_{50} and IC_{90} values was performed using XLfit 4® software with other data and statistical analysis performed using Microsoft Excel® software.

4:4 RESULTS

Comparison of incubation at 25°C and 37°C in the MTS assay and the optimisation of T. cruzi epimastigote concentrations

In order to determine the optimal concentration of *T. cruzi* epimastigotes for use *in vitro* for viability assays, an experiment was designed to assess the ability of the novel tetrazolium salt, MTS, to be reduced by different concentration of parasites at different incubation temperatures. Whilst the incubation temperature for MTS reduction identified by the manufacturer is 37°C, *T. cruzi* epimastigotes are maintained *in vitro* at 25°C. A comparison was made between the incubation of MTS at 37°C and 25°C to determine if this final incubation step of 37°C in the presence of MTS would affect the viability of *T. cruzi* epimastigotes over the time required to reduce formazan by producing a colour change which was measurable at 492nm. In this series of experiments it could be seen that increasing the concentration of parasites in the well from 5 x 10^3 ml^{-1} to 1 x 10^7 ml^{-1} led to an increase in MTS reduction at both incubation temperatures of 37°C (Figure 4.1) and 25°C (Figure 4.2) following incubation of parasites alone at 25°C for 24 h.

The corrected maximum absorbance for parasites incubated with MTS at 37°C ranged from 0.354 ± 0.023 after 1 h to 0.821 ± 0.06 after 4 h with a concentration of $1x10^{-7}$ ml^{-1} epimastigotes (Figure 4.1). This did not significantly vary from when the parasites were incubated with MTS at 25°C from 1.5 h to 6 h (0.342 ± 0.012 to 0.729 ± 0.045; Figure 4.2) except when comparing both the 3 h and 4 h incubation points. In each case, incubation at 37°C significantly increased absorbance and therefore MTS reduction when compared with incubation at 25°C (3 h incubation = 37°C 0.709 ± 0.046 *vs*. 25°C 0.513 ± 0.01, $P < 0.01$; 4 h incubation = 37°C 0.821 ± 0.06 *vs*. 25°C 0.672 ± 0.047, $P < 0.05$). From this it could be determined that a more rapid response could be obtained by incubating the epimastigotes in the presence of MTS at 37°C rather than for the corresponding time at 25°C, with no loss of viability as seen by the continued metabolic reduction of MTS. To obtain an absorbance equal to 4 h incubation at 37°C one had to incubate the parasites with MTS for 6 h at 25°C.

It was observed that the lower limit of detection for the absorbance of reduced MTS at 37°C corresponded to 1 x 10^5 ml^{-1} viable epimastigotes (Figure 4.1) but a longer

incubation period for MTS at 25°C and more viable epimastigotes (5 x 10^{-5} ml^{-1}, Figure 4.2) were required before detection. A concentration of 1 x 10^{7} ml^{-1} epimastigotes represented the upper detection limits for both incubation temperatures whereby differences in viability could be observed (such as decreases and increases in parasite numbers) and detected readily at both 3 h 37°C and 6 h 25°C incubation. This concentration was used for all future *in vitro* viability experiments.

Although a 3 h incubation of MTS at 37°C showed no significant difference between the 4 h incubation (Figure 4.1, $P = 0.063$) and could be used in viability experiments, it was decided that as epimastigotes undergo physical changes at 37°C in the human host, 37°C would not be used as an MTS incubation temperature. Instead, 25°C was used with the lengthier incubation of 4 h in the MTS assay as this incubation temperature and time showed no significant difference between the absorbance's detected for 1 x 10^{7} ml^{-1} epimastigotes at 37°C with 3 h MTS incubation (25°C, 4 h = 0.672 ± 0.047; 37°C 3 h = 0.709 ± 0.047, $P = 0.378$).

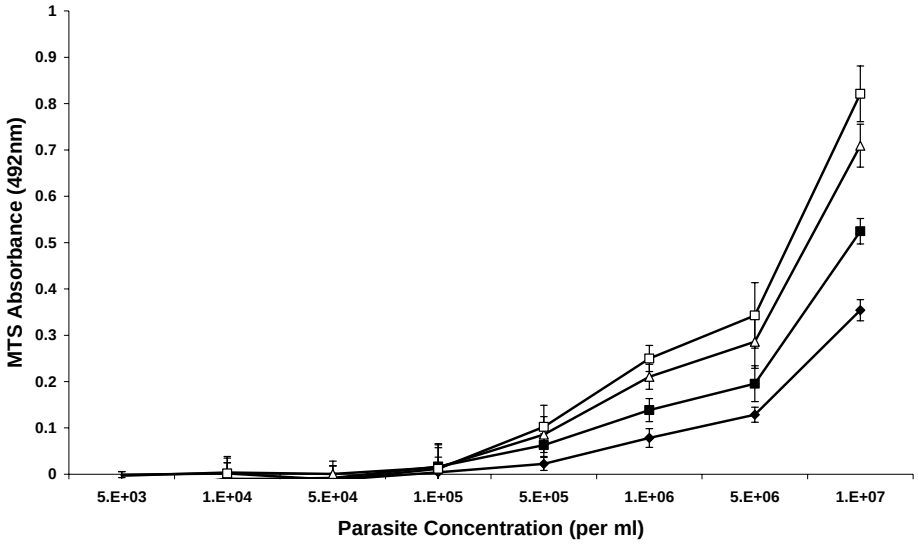


Figure 4.1 Assessment of epimastigote viability and optimal concentration using MTS at 37°C.
Different concentrations of *T. cruzi* epimastigotes were incubated in 96 well microtitre plates in complete medium for 24 h at 25°C. On completion of incubation 20µl of MTS were added to each well of the plate and then incubated in the dark for (◆) 1, (■) 2, (△) 3, or (□) 4 h at 37°C.

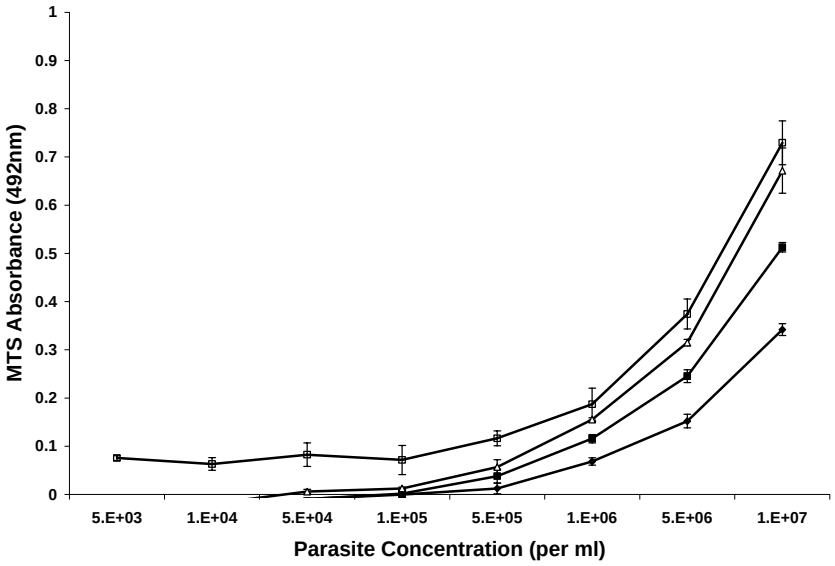


Figure 4.2 Assessment of epimastigote viability and optimal concentration using MTS at 25°C.
Different concentrations of *T. cruzi* epimastigotes were incubated in 96 well microtitre plates in complete medium for 24 h at 25°C. On completion of incubation 20µl of MTS were added to each well of the plate and then incubated in the dark for (◆) 1.5, (■) 3, (△) 4, or (□) 6 h at 25°C.

T. cruzi epimastigote morphological alterations (TBMM assay)

Epimastigotes of *T. cruzi* were incubated in complete Optimem for 24 h at 25°C and light microscopy images were taken. Reference drugs were unavailable for comparison testing and so an alternative method was employed to show epimastigote death under Trypan blue staining. The method selected was heat killing at 56°C for 10 min. Viable epimastigotes were motile, possessing a single long whip-like flagellum and a slender oval cell body which excluded Trypan blue (Figure 4.3 A). Heat killing of epimastigotes led to internalisation of the flagellum, rounding up and swelling of the cell body, accompanied by uptake of Trypan blue (Figure 4.3 B). Some epimastigotes remained oval shaped and elongated, with the flagellum retaining its full length, but were non-motile and appeared in a comma shaped configuration (Figure 4.3 B).

Effects of ethanol on T. cruzi viability

Incubation of 1 x 10^7 ml^{-1} *T. cruzi* epimastigotes in the presence of ethanol at 0.06%, did not significantly reduce their viability over a 24 h period. This was demonstrated using two methods; the MTS assay and TBMM assay. Ethanol treated and untreated epimastigotes both caused the reduction of formazan in the MTS assay to similar levels as indicated by the absorbance at 492nm (ethanol = 0.827 ± 0.06 *vs*. untreated = 0.821 ± 0.03; $P > 0.05$). This similarity was also demonstrated in the TBMM assay where no significant difference in their viability between ethanol treatment and untreated epimastigotes could be determined (ethanol = 98.02 ± 1.4% *vs*. untreated = 98.04 ± 1.5%; $P > 0.05$).

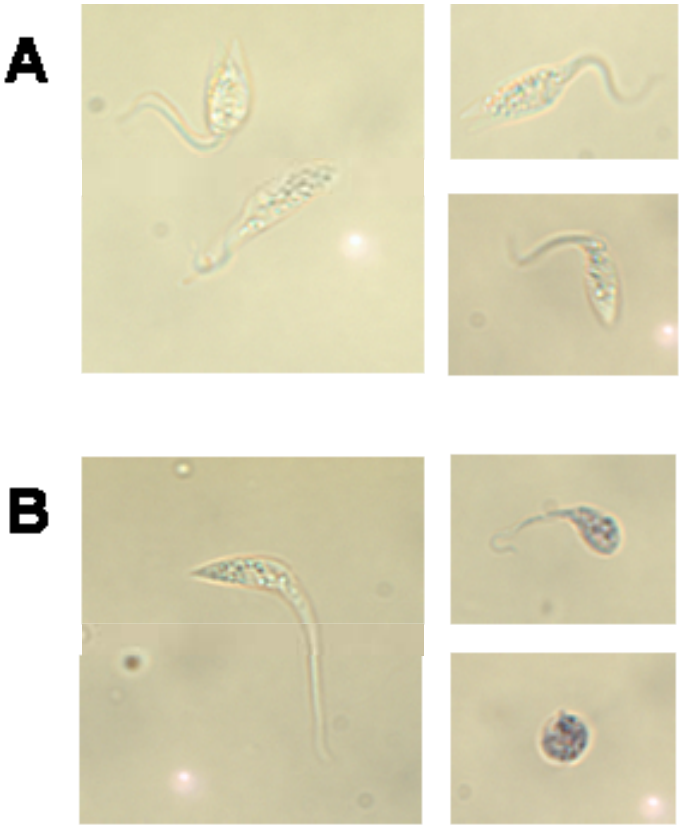


Figure 4.3 Viable and non-viable epimastigotes of *Trypanosoma cruzi* as assessed by TBMM.
Epimastigotes of *T. cruzi* were incubated in complete Optimem at 25°C for 24 h and then aliquots were either mixed directly with an equal volume of Trypan blue (**A**) or treated with 56°C for 10 min prior to mixing with Trypan blue (**B**) and then viewed under a light microscope (total mag. X1250). Images are from representative samples. Photographs were taken (copyright© SPDL) using a ColorView Soft Imaging System MTV-3 camera and analySIS® software.

Incubation of T. cruzi epimastigotes with PEOs

Screening of PEOs

A range of 12 PEOs were incubated with *T. cruzi* epimastigotes for 24 h at 25°C. When screened at a final concentration of 0.02% in each well, each PEO killed 100% of epimastigotes as assessed by Trypan blue inclusion / exclusion (Figure 4.4 A). When the MTS assay was used to investigate viability, all PEOs with the exception of myrrh, caused little or no reduction of formazan by the epimastigotes, indicating that they were non-viable (Figure 4.4 B). The formazan reductive activity observed with myrrh treated trophozoites was slight, but significantly different from other PEO treated parasites ($P < 0.05$). Representative examples of PEO influence on morphology are given in Figure 4.5. Both untreated and ethanol treated epimastigotes show typical spindle shaped cell bodies with a single long flagellum (Figure 4.5 A & B). All the oils caused the epimastigotes to round up and include Trypan blue dye, indicating non-viability. The parasite cell body, apart from rounding up, also significantly contracted in length and area size, with the exception of myrrh (Table 4.2, $P < 0.05$). In many cases a shortened flagellum free of the undulating membrane was present (Figure 4.5 D – G, I, K, M & N). An absence of a flagellum occurred with oils of sweet fennel, patchouli, myrtle and thyme (Figure 4.5 C, H, J & L respectively). The greatest change in morphology occurred with oils of myrtle and thyme, with these oils reducing epimastigotes to a non-motile, unflagellated and poorly defined morphology (Figure 4.5 J & L, respectively) over 24 h incubation.

Table 4.2 Effect of PEO treatment on *T. cruzi* epimastigote size.

	Epimastigote Cell Body Measurement		
PEO	**Length (µm)**	**Breadth (µm)**	**Area (µm^2)**
Control	8.94 ± 1.77	3.36 ± 0.34	20.52 ± 4.77
Amyris	4.44 ± 0.95	3.42 ± 0.82	10.82 ± 3.90
Buchu	5.05 ± 0.73	3.75 ± 0.61	13.84 ± 2.91
Elemi	4.71 ± 0.69	3.49 ± 0.65	10.16 ± 2.35
Geranium	4.18 ± 0.62	3.15 ± 0.58	10.42 ± 2.42
Lavender	4.24 ± 0.95	3.42 ± 0.82	10.82 ± 3.90
Marjoram	4.66 ± 0.61	3.59 ± 0.86	12.72 ± 2.49
Myrrh	5.65 ± 1.04	4.18 ± 0.72	17.57 ± 6.10
Myrtle	4.42 ± 0.75	3.25 ± 0.40	10.68 ± 2.46
Palmarosa	4.48 ± 0.90	3.53 ± 0.76	11.44 ± 4.63
Patchouli	4.35 ± 0.42	3.31 ± 0.42	10.44 ± 3.44
Sweet Fennel	4.55 ± 0.73	3.81 ± 0.76	13.25 ± 4.12
Thyme	4.94 ± 0.51	3.75 ± 0.85	15.63 ± 3.78

Parasites were incubated at 25°C in 96 well microtitre plates in the presence or absence of different PEOs at a final concentration 0.02% in the well. Epimastigote viability was assessed by TBMM under light microscopy using a total magnification of x1250. Images of epimastigotes were taken using a ColorView Soft Imaging System MTV-3 camera and analysed using analySIS® software. Measurements from 10 epimastigotes were taken for each PEO.

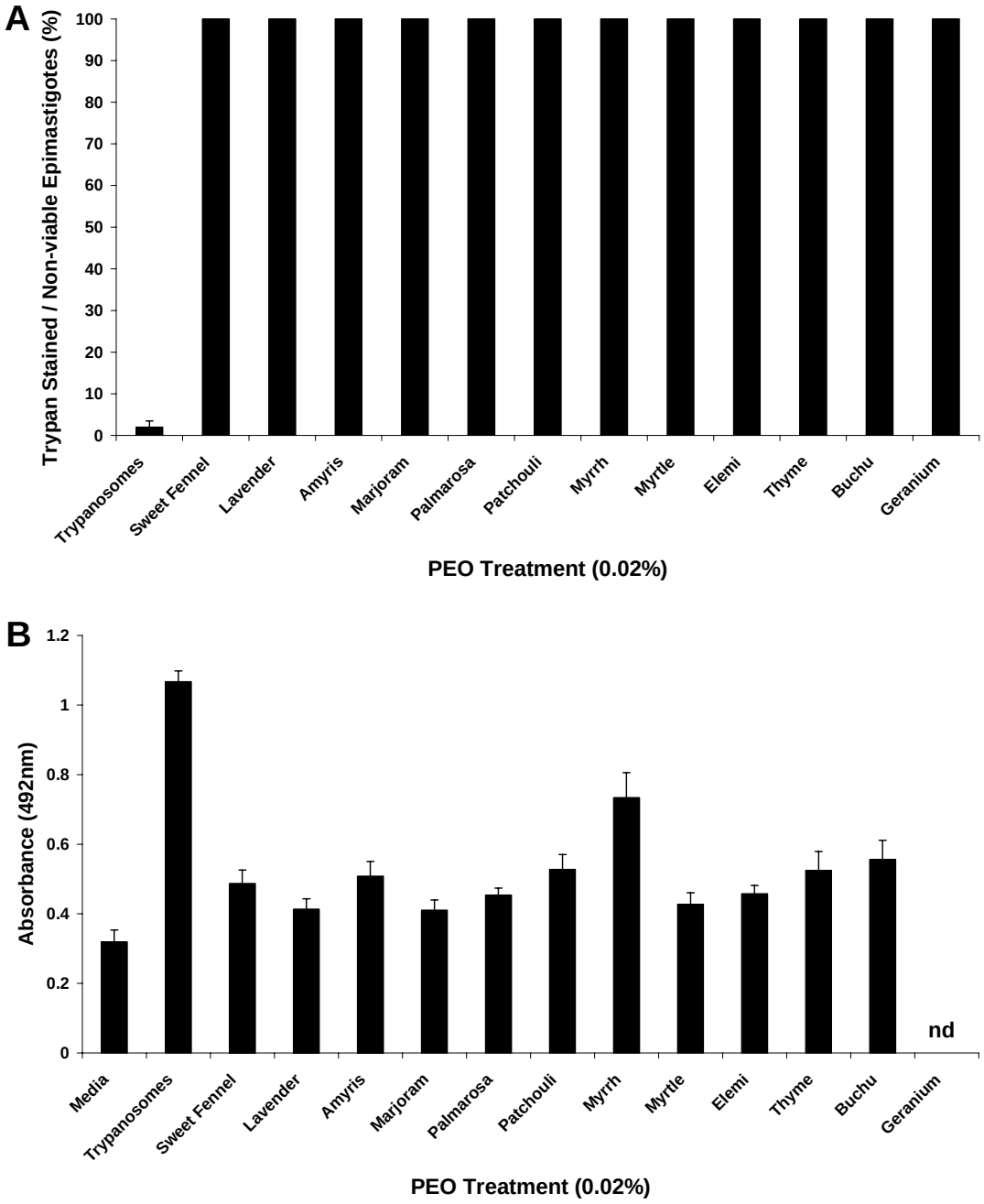


Figure 4.4 Incubation of *T. cruzi* epimastigotes *in vitro* with various PEOs.
Parasites were incubated at 25°C in 96 well microtitre plates in the presence or absence of different PEOs at a final concentration 0.02% in the well or ethanol at a final concentration of 0.06%. **A**) Parasite viability was assessed by TBMM under light microscopy or **B**) MTS solution (20 µl per well) added directly to the wells following PEO exposure and incubated for 4h at 25°C in the dark before the absorbance at 492nm was measured. Geranium oil was not available when this assay was performed (nd).

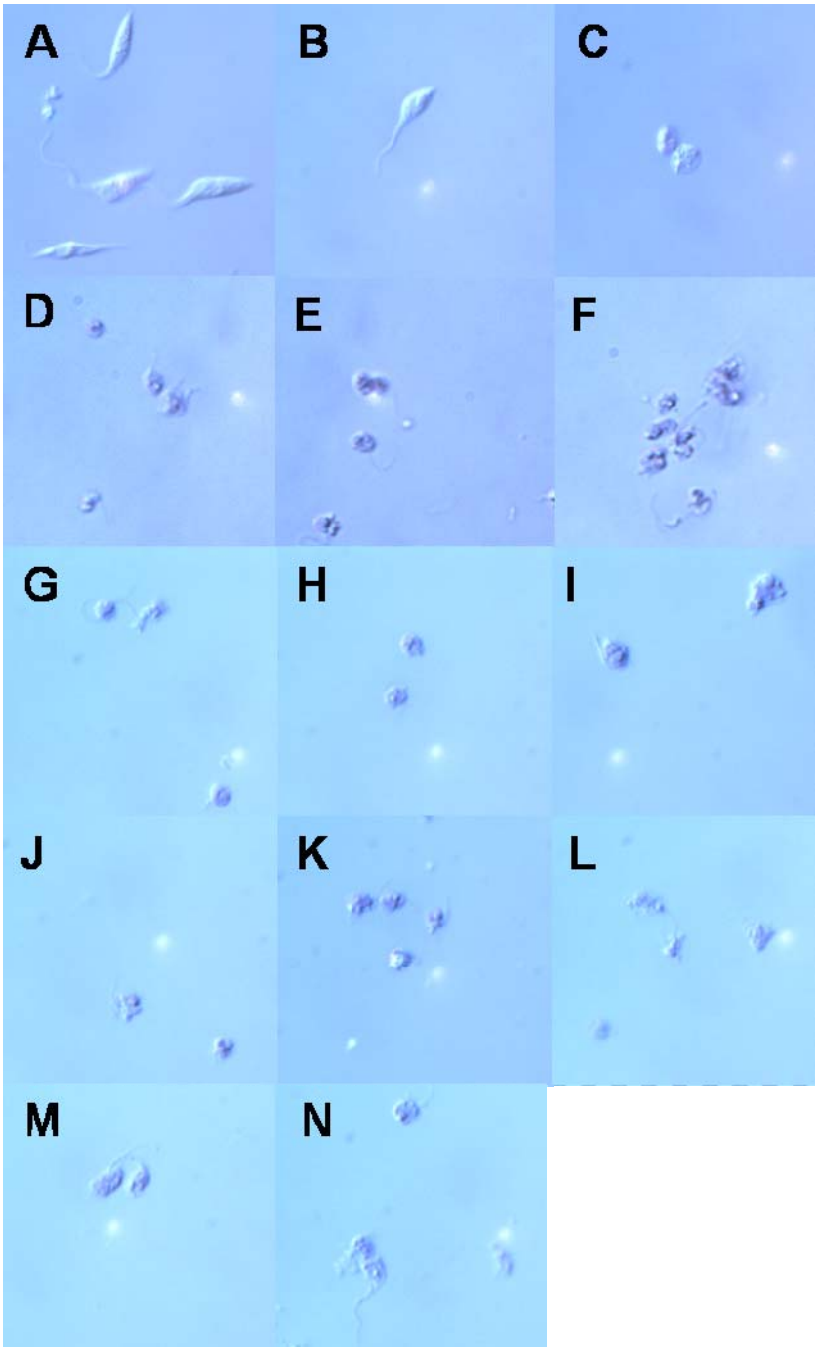


Figure 4.5 Incubation of *T. cruzi* epimastigotes *in vitro* with various PEOs showing their effect on morphology.
Parasites were incubated at 25°C in 96 well microtitre plates in the presence or absence of different PEOs at a final concentration 0.02% in the well or ethanol at a final concentration of 0.06%. **A**) epimastigotes alone, **B**) ethanol treated, **C**) sweet fennel, **D**) lavender, **E**) amyris, **F**) marjoram, **G**) palmarosa, **H**) patchouli, **I**) myrrh, **J**) myrtle, **K**) elemi, **L**) thyme, **M**) buchu and **N**) geranium. Parasite viability was assessed by Trypan blue inclusion/exclusion under light microscopy. Representative photographs of epimastigotes using a total magnification of x1250 under Nomarski DIC. Photographs were taken (copyright© SPDL) using a ColorView Soft Imaging System MTV-3 camera and analySIS® software.

Titration of PEOs

As all the PEOs at a final concentration of 0.02% caused 100% killing of epimastigotes, a PEO titration was determined. Doubling dilutions of all PEOs were incubated with epimastigotes for 24 h at 25°C, beginning with the concentration used for screening (0.02%).

The epimastigotes were sensitive to all of the PEOs at concentrations below 0.02% with the lowest MIC being recorded for myrtle and elemi (0.00125%; Figure 4.6 and Table 4.3). All other oils had MICs of 0.01% (thyme and buchu), 0.005% (lavender, amyris, marjoram, palmarosa and patchouli) and 0.0025% (sweet fennel, myrrh and geranium; Figure 4.6 and Table 4.3).

Lowering the concentration of myrtle and elemi oil below the MIC to 0.000625% reduced antitrypanosomal effects (from 100% epimastigote killing to 97 – 97.7%), but this was not found to be significant ($P > 0.05$). The same level of effect (97.6 ± 1.9%; Figure 4.6) was maintained with myrtle at a concentration of 0.000313%, but not with elemi (40.6 ± 32.9%; Figure 4.6). However, once myrtle oil was diluted to 0.000156% there was no significant difference in epimastigote viability between the untreated control and the 0.000156% myrtle oil treated epimastigotes (control = 6.5 ± 6.5% *vs*. 0.000156% myrtle = 8.6 ± 5.0%, $P > 0.05$).

Whilst reference drugs were unavailable for use in these experiments, other investigators have been able to source benznidazole and IC_{50} values of 7.4 – 50 µg ml^{-1} have been quoted with *T. cruzi* epimastigotes *in vitro* (del Olmo *et al*. 2001; Sepúlveda-Boza & Cassels 1996). In order to compare the PEOs with benznidazole the percentage concentrations had to be converted into µg ml^{-1} as described in Chapter 2.

All PEOs tested were more effective than benznidazole in killing epimastigotes(IC_{50} = 2 – 39 µg ml^{-1} [0.0002 – 0.0039%], Table 4.3). Both myrtle and elemi exhibited the lowest IC_{50} results, being 2 and 3 µg ml^{-1} respectively (Table 4.3). These figures are lower than that of benznidazole as cited in the literature (del Olmo *et al*. 2001;

Sepúlveda-Boza & Cassels 1996) and suggest that both these oils are highly antitrypanosomal.

When treated with 0.02% of myrtle or elemi oil, changes in parasite morphology included: rounding up of the cell body and an absence of a flagellum (Figure 4.7 A & B far left panel). As the concentration of PEO decreased the morphology of the treated epimastigotes changed from being rounded, lacking a visible flagellum and stained with Trypan blue to becoming typically spindle shaped with a highly motile flagellum being observed with a concentration of 0.000156% oil (Figure 4.7 A & B viewed left to right). These changes in morphology observed for the 2 oils demonstrate the rapid switch between killing and survival of the epimastigotes at concentrations just below the MIC shown in figure 4.6.

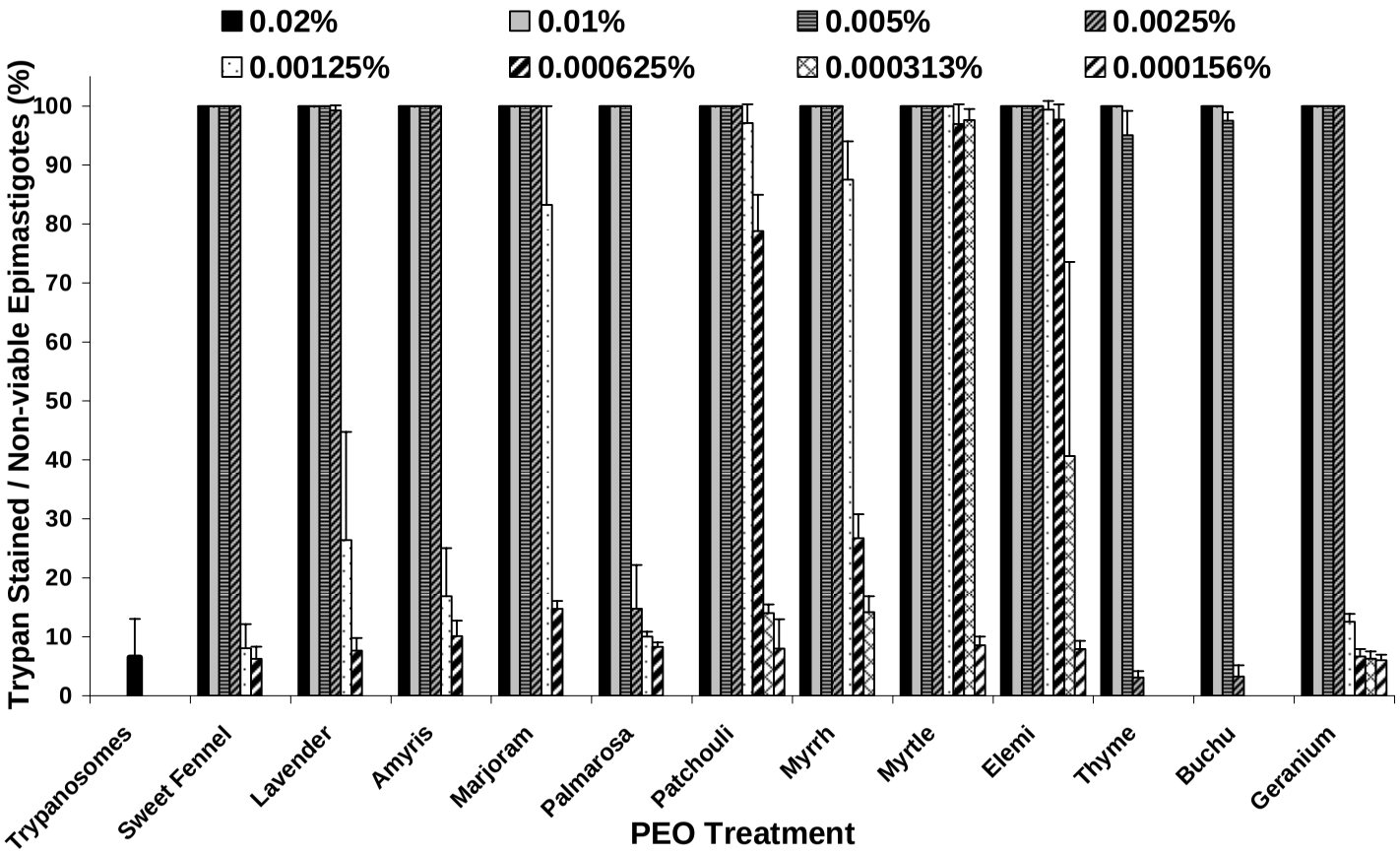


Figure 4.6 Titrations of all PEOs investigated with *T. cruzi* epimastigotes *in vitro*.
Parasites were incubated in the presence or absence of doubling dilutions of plant oils. Final concentrations used in each well started at 0.02% with doubling dilutions of 0.01%, 0.005%, 0.0025%, 0.00125%, 0.000625%, 0.000313% and 0.000156%. Parasite viability was assessed by TBMM under light microscopy.

Table 4.3 Inhibition of *T. cruzi* epimastigote survival by different PEOs

	INHIBITORY CONCENTRATION					
PEO	**MIC[a]**	**MIC[b]**	**IC_{90}[a]**	**IC_{90}[b]**	**IC_{50}[a]**	**IC_{50}[b]**
Buchu	0.01	100	0.0045	45	0.0037	37
Thyme	0.01	100	0.0047	47	0.0039	39
Amyris	0.005	50	0.0022	22	0.0016	16
Lavender	0.005	50	0.002	20	0.0015	15
Marjoram	0.005	50	0.0014	14	0.0009	9
Palmarosa	0.005	50	0.0044	44	0.0033	33
Patchouli	0.005	50	0.0007	7	0.0005	5
Geranium	0.0025	25	0.0021	21	0.0017	17
Myrrh	0.0025	25	0.0014	14	0.0008	8
Sweet Fennel	0.0025	25	0.002	20	0.0017	17
Elemi	0.00125	12.5	0.0005	5	0.0003	3
Myrtle	0.00125	12.5	0.0003	3	0.0002	2

[a] Concentrations are expressed as a percentage v/v.
[b] Concentrations are expressed as µg ml^{-1} where 0.001% = 10 ppm = 10 µg ml^{-1}.
MIC = the minimum concentration required to inhibit the growth / survival of 100% epimastigotes.
IC_{90} and IC_{50} = the concentration required to inhibit 90% or 50% of epimastigotes respectively.
Inhibition was assessed using TBMM.

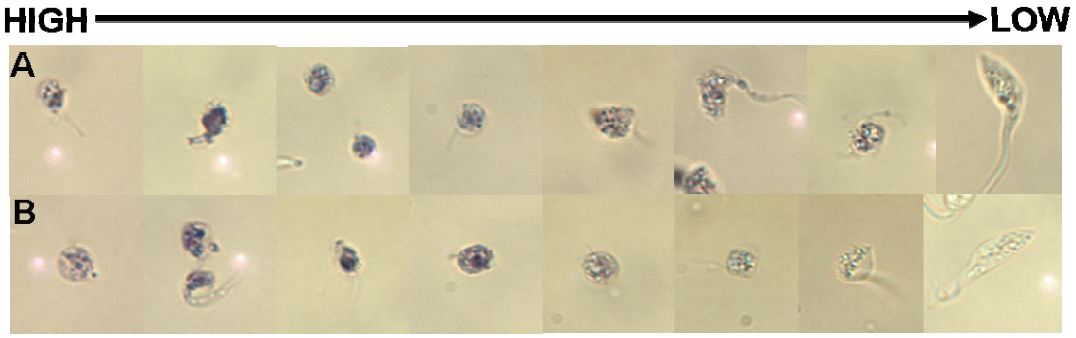


Figure 4.7 Titrations of myrtle and elemi PEOs investigated with *T. cruzi* epimastigotes *in vitro* demonstrating morphological alterations.
Parasites were incubated in the presence or absence of doubling dilutions of **A**) myrtle oil or **B**) elemi oil. Final concentrations used in each well started at 0.02% (first panel on the left hand side) with doubling dilutions (0.01%, 0.005%, 0.0025%, 0.00125%, 0.000625%, 0.000313% and 0.000156%) progressing from left to right (**HIGH** to **LOW**). Parasite viability was assessed using TBMM. Representative photographs of epimastigotes using a total magnification of x1250. Photographs were taken (copyright© SPDL) using a ColorView Soft Imaging System MTV-3 camera and analySIS® software.

Incubation of T. cruzi epimastigotes with PEO constituents

Considering that myrtle oil was the only oil able to sustain 97.6 ± 1.9% epimastigote killing down to a concentration of 0.000313% over 24 h, the activity of this PEO was further investigated. As previously shown in Chapter 2 and Appendix 3, myrtle oil consists of several constituents, with its main constituents being α-pinene (51.72%) and eucalyptol (22.35%). Given this information, a titration of each constituent either singly or in combination was carried out to determine if they were responsible for the observed epimastigote killing. The constituents were used at concentrations equivalent to that to be found in the whole oil such that the starting concentration of α-pinene was 0.01% (equivalent of 0.02% whole oil) and eucalyptol was 0.0044%.

When used at 0.01%, α-pinene alone reduced epimastigote viability by 100% (Figure 4.8). A concentration dependent decrease in α-pinene efficacy was also demonstrated. At 0.0025%, α-pinene did not reduce the viability of epimastigotes. This concentration of α-pinene was the equivalent of 0.005% myrtle oil, which has previously been demonstrated to kill 100% of epimastigotes in the same incubation time (Figures 4.6 and 4.9). The secondary constituent of myrtle oil, eucalyptol, had no significant effect on epimastigote viability (Figure 4.8, $P > 0.05$).

A concentration dependent increase in antitrypanosomal activity was demonstrated when these 2 constituents were used in combination. There was no significant reduction in epimastigote viability until a myrtle oil equivalent concentration of 0.005% was reached. At this concentration the effect of 0.0025% α-pinene + 0.0011% eucalyptol was more than additive and most likely to be synergistic (Figure 4.8). If the activity was additive, then epimastigote mortality would be 4.3 ± 3.9%. However, when used in combination, these 2 constituents caused 24.6 ± 15.2% death of epimastigotes, significantly greater than their additive activities ($P < 0.05$) and a 5.7 fold increase on the additive value. The activity of α-pinene and eucalyptol combined was always greater than that of α-pinene alone, but was only significantly higher at a myrtle equivalent concentration of 0.005%.

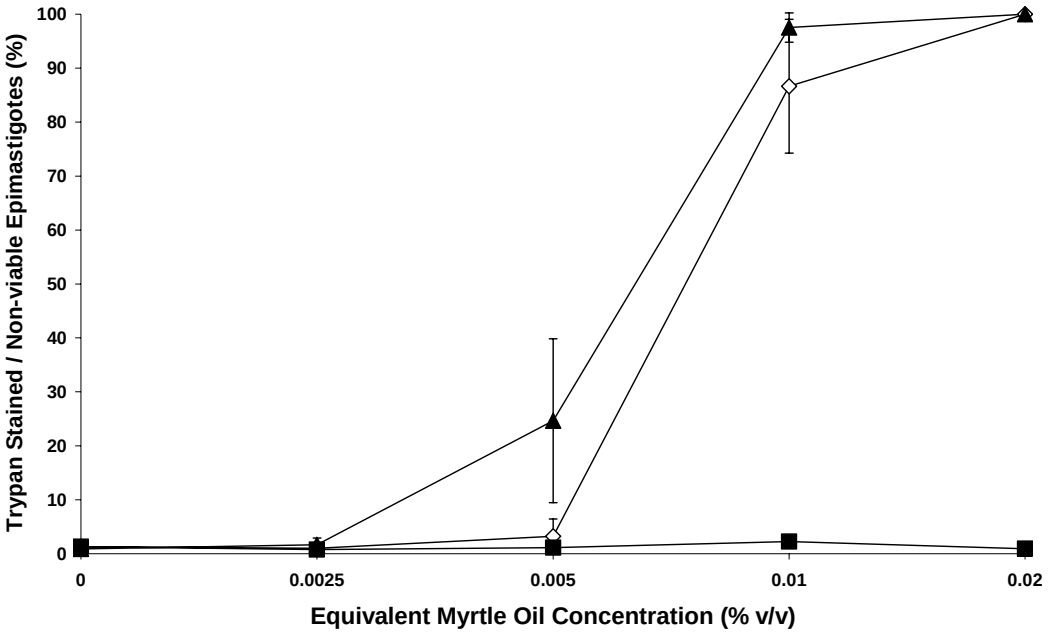


Figure 4.8 Titration of the 2 major constituents of myrtle oil investigated with *T. cruzi* epimastigotes *in vitro*.
Parasites were incubated in the presence or absence of doubling dilutions of α-pinene and eucalyptol on their own or in combination. The starting concentration used was the equivalent concentration to be found in 0.02% of whole oil. α-pinene [◇] would therefore start at a concentration of 0.01% and eucalyptol [■] at 0.0044%. These 2 constituents were also used in combination [▲] at 0.01% for α-pinene and 0.0044% for eucalyptol. Parasite viability was assessed by using TBMM.

Incubation of T. cruzi epimastigotes with PEOs over a 2 h time course

Whilst PEO treated epimastigotes were being incubated at either their screening concentrations or MICs, routine observations after 2 h at 25°C indicated that morphological changes were occurring with the treated epimastigotes. In order to quantify this observation, a 2 h time course was performed at PEO MIC levels with epimastigotes *in vitro*. Of the 12 PEOs investigated, 9 reduced epimastigote viability by 100% after 2 h incubation (Figure 4.9). Oils of lavender, marjoram and myrtle did reduce epimastigote viability, but to a lesser degree than described for the other 9 PEOs (99.3 ± 0.8%, 99.8 ± 0.4% and 99.0 ± 1.1% respectively). This reduction in the activity of the oils was slight but was not significant ($P > 0.05$) for lavender and marjoram oils and for myrtle oil ($P = 0.0502$). These results would indicate that PEOs killing of epimastigotes can occur within 2 h.

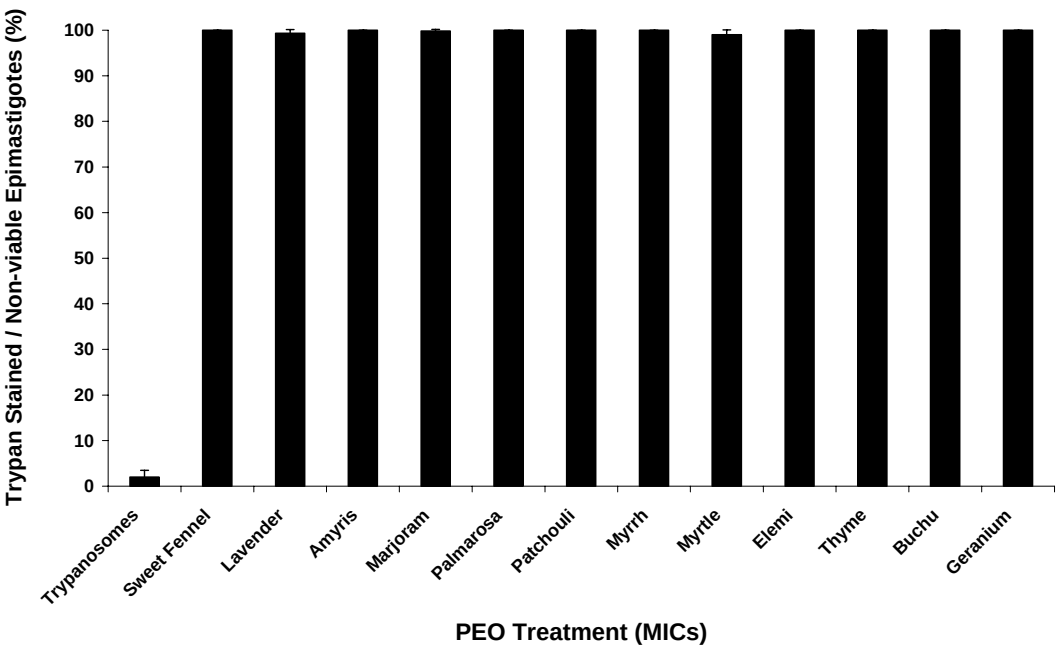


Figure 4.9 Incubation of *T. cruzi* epimastigotes *in vitro* with various PEOs at their MICs for 2 h.

Parasites were incubated at 25°C for 2 h in 96 well microtitre plates in the presence or absence of different PEOs at their MICs. These MICs are as follows: sweet fennel (0.0025%), lavender (0.0025%), amyris (0.0025%), marjoram (0.0025%), palmarosa (0.05%), patchouli (0.0025%), myrrh (0.0025%), myrtle (0.00125%), elemi (0.00125%), thyme (0.01%), buchu (0.01%) and geranium (0.0025%). Parasite viability was assessed using TBMM.

4:5 DISCUSSION

This series of experiments was designed to (i) determine working concentrations of *T. cruzi* epimastigotes and diluents for use in this project and (ii) to identify whether PEOs could influence *T. cruzi* epimastigote viability *in vitro* over time.

Light microscopy was used to identify morphological changes in the epimastigotes, such as absence of a flagellum and rounding up of the cell body. As identified in Chapter 2, this is the most commonly utilized test for cell viability (Eisenbrand *et al*. 2002; Freshney 1994; Patterson 1979; Pappenheimer 1917).

A colourmetric biochemical assay was also used to assess viability. The MTS cell proliferation assay is used to identify the cytotoxic potential of a sample. This assay measures (by absorbance) the formation of a soluble formazan product which is directly proportional to the number of live cells in the investigated sample. The influence of incubation time on absorbance values generated with the MTS assay has been previously reported (Cory *et al*. 1991; Buttke, McCubrey & Owen 1993), where a linear relationship between incubation time and absorbance for short incubation times of up to 5 h was demonstrated. An advantage of MTS over the original MTT assay is that the formazan crystals of MTS are soluble in culture medium and don't require solublisation. Formazan crystals of MTT are insoluble in culture medium and require the addition of SDS or DMSO for solublisation whereas the MTS does not need a solublisation step. This difference of an additional step for solublisation may be small, but it can increase the length of time for an assay to be completed, its individual cost and accuracy due to increased manipulation of samples. Since MTS has been assessed by others to be faster than, simpler than and just as accurate as MTT (Berg *et al*. 1994; Cory *et al*. 1991), this method was employed for the assessment of *T. cruzi* epimastigote viability in conjunction with Trypan blue inclusion/exclusion, motility and morphology.

To date much of the antimicrobial and antiparasitical work involving plants has revolved around crude extracts (methanolic, ethanolic or water), with very little work regarding PEOs being investigated. In the last 17 years, 199 papers have been

published concerning the trypanocidal effects of plants and their extracts (PubMed search performed 10th January 2008) but, of these papers, only 5 concerned essential oils.

TTO, lemon balm, thyme, peppermint, basil, *Strychnos spinosa*, yarrow, clove, lemongrass and oregano oils have all been investigated for their *in vitro* trypanocidal properties (Chapter 1, Table 1.1; Mikus *et al*. 2000; Hoet *et al*. 2006; Santoro *et al*. 2007a, 2007b and 2007c). In the experiments of Mikus *et al*. (2000) lemon balm, peppermint, thyme and TTO demonstrated greater toxicity to *T. brucei* trypomastigotes than to human HL-60 cells *in vitro*. *Strychnos spinosa* oil has shown activity against *T. brucei* trypomastigotes that was not selective for the parasite alone, being toxic to J774 macrophages, but 2 of its constituents, E-nerolidol and linalool, were more effective against the parasite than the PEO (*Strychnos spinosa* IC_{50} = 13.5 μg ml^{-1}; E-nerolidol IC_{50} = 1.7μg ml^{-1}; E-nerolidol IC_{50} =2.5 μg ml^{-1}; Hoet *et al*. 2006). These 2 constituents were also found to have a more selective activity to the parasite than to the J774 macrophage (SI = 35.7 and > 40 respectively). Growth inhibition of *T. cruzi* epimastigotes and trypomastigotes by thyme and its constituent thymol has been demonstrated with cytoplasmic swelling occurring (Santoro *et al*. 2007a). In addition the oils of basil, clove, lemon grass, oregano and yarrow have also been tested against *T. cruzi* epimastigotes and trypomastigotes and exhibit a range of IC_{50} from 77 – 145.5 μg ml^{-1} for epimastigotes and 15.5 – 467.5 μg ml^{-1} for trypomastigotes (Santoro *et al*. 2007a, 2007b and 2007c). Here, 12 PEOs were investigated. This increased the total number of PEOs evaluated for trypanocidal action in the literature from 10 to 22.

A concentration of epimastigotes was determined for use using the MTS assay. The requirements are that there must be sufficient to be detectable by the assay at both low and high viability levels. A concentration of 1 x 10^7 ml^{-1} epimastigotes added to the wells of a 96 well microtitre plate was found to elicit an absorbance level (~0.800) which could be readily observed after 24 h incubation, allowing increases and decreases in cell numbers to be determined. Also determined was a time and temperature dependant increase in absorbance. Increasing the incubation temperature

from 25°C to 37°C following addition of MTS allowed for more rapid formazan reduction to take place. Incubation at 37°C for the time of assay development (up to 4 h) had no adverse effects on the epimastigote viability, as assessed by formazan reduction, where after 3 h of incubation at 37°C, a level of absorbance was found which could be used in experiments. Whilst epimastigote viability was not affected at 37°C, there is the potential for the transformation of epimastigotes to trypomastigotes at this higher temperature. For this reason, an incubation of up to 6 h at 25°C was used.

In order to accurately assess the activity or effectiveness of a test sample a reference compound is used to compare activities / effects. Both benznidazole (syn. Rochagan® and Radanil®) and nifurtimox (syn. Lampit®) are the recognised drugs of choice for American trypanosomiasis, with benznidazole regarded as the first choice. However, it is difficult to obtain. Production of benznidazole is intermittent, as the production and licence of the drug has been transferred from Roche to the government of Brazil. Availability is limited due to problems of sourcing the main ingredients for drug production. Difficulties in the acquisition of nifurtimox is due to its production being suspended by Bayer in 1997 and then restarted on a limited scale for the treatment of African trypanosomiasis in 2000 on a 'demand' basis, i.e. if there is enough demand the company will continue its production (Jannin & Villa 2007). At the time of this project, neither drug was unavailable for purchase.

PEOs required to be diluted in a non-aqueous diluent and since, in previous experiments using *G. duodenalis* trophozoites, ethanol had demonstrated no adverse effect on trophozoite viability (Chapter 2), this diluent was tested with *T. cruzi* epimastigotes. Ethanol when used at a concentration of 0.06% demonstrated no effect on epimastigote viability and so was chosen as the diluent for PEOs in experiments.

When used at a final concentration of 0.02%, all PEOs investigated reduced epimastigote viability by 100%, causing the rounding up of epimastigotes *in vitro* when compared to untreated epimastigotes. The epimastigote killing was confirmed

using the MTS assay, where a lack of colour change in the assay indicates the prevention of biochemical reduction of MTS to formazan. This can indicate that the function of the single mitochondria present in this organism was inhibited.

Unfortunately, at this point in the project, the parasite stocks became problematic to culture in sufficient quantity for all experiments. The MTS assay experiments were performed in the latter half of the project after the Trypan blue data had been collected and, therefore, much of the TBMM data obtained could not be corroborated with the MTS assay.

The greatest observed effect of PEOs was on the morphology of epimastigotes. The rounding up of the epimastigotes (Figure 4.5) suggest that the cellular membrane remains intact and that perhaps the oils affect the cytoskeleton rather than damaging the cell membrane, or perhaps change the osmoregularity of the cell, as this would tend to cause swelling of the cell body seen in the *G. duodenalis* experiments (Chapter 2). Also seen was the reduction in area of the epimastigote cell body by up to 50% (Table 4.2). Further experiments using SEM or TEM would help in the morphological analysis of PEO treated epimastigotes and give insights into the mechanism of action of PEOs by defining any morphological alterations. Such analysis has been carried out by Santoro *et al*. (2007a), where trypanosomes treated with oregano and thyme oil, demonstrated rounding of the cell body with little alteration at the plasma membrane using SEM. TEM studies, however, showed cytoplasmic swelling with occasional morphological alteration in the plasma and flagellar membrane such as blebbing.

The PEOs investigated had IC_{50}s ranging from 2 – 39 μg ml^{-1} ([0.0002 – 0.0039%], Table 4.3). As reference drugs were unavailable for use in these experiments, other investigators found that benznidazole IC_{50} levels were in the range 2.82 – 16.9 μM (0.73 – 4.4 μg ml^{-1}) (Batista *et al*. 2008; Jimenez-Ortiz *et al*. 2005; Villarreal *et al*. 2004; del Olmo *et al*. 2001), with concentrations up to ~50 μg ml^{-1} being quoted with *T. cruzi* epimastigotes *in vitro* (Sepúlveda-Boza & Cassels 1996). Both myrtle and elemi oils exhibited the lowest IC_{50} data, being 2 and 3 μg ml^{-1} respectively (Table

4.3). These figures are within the quoted range for benzidazole and suggest that these PEOs are as potent as the reference drugs. These 2 oils may have further potential as a source of novel antitrypanosomal drugs.

There is evidence in the literature to show that the constituents of essential oils exert trypanocidal actions. The terpene, terpinen-4-ol, from TTO oil has a high selectivity of 1000 (i.e. being 1000 times more selective against the parasite than human HL-60 cells), which is within the range of commercially available drugs such as Suramin (Mikus *et al*. 2000). Further, terpenic compounds from *Strychnos spinosa* (E-nerolidol and linalool) have demonstrated antitrypanosomal activity, inhibiting the growth of the bloodstream form of *T. brucei in vitro* (Hoet *et al*. 2006). In the experiments of Hoet *et al*. (2006) and Mikus *et al*. (2000), the terpenic compounds were found to have a greater affect than that of the whole oil.

Since myrtle oil was the most effective oil to be tested in this project, an investigation into its major constituents was carried out. It was determined that the monoterpenes, α-pinene and eucalyptol comprised 51.72% and 22.25% of myrtle oil respectively. At concentrations found in 0.02% of the whole oil, α-pinene (0.01%) was able to reduce to 0% the number of viable epimastigotes as assessed by TBMM, whereas eucalyptol (0.0044%) failed to have any apparent effect on viability (Figure 4.8). This result indicated that α-pinene was the active constituent in myrtle oil. When this constituent was titrated, a concentration dependent increase in viability was demonstrated, so at 0.0025% (myrtle oil equivalent concentration of 0.005%) there was 100% viability (Figure 4.8). When 0.0011% eucalyptol was used in conjunction with a corresponding concentration of α-pinene (0.0025%) to be found in 0.005% myrtle oil, an increase in epimastigote killing was demonstrated when compared to either eucalyptol or α-pinene alone (eucalyptol 0.0011% = 1.1 ± 0.72%; α-pinene 0.0025% = 3.2 ± 3.2%; α-pinene + eucalyptol = 24.6 ± 15.2%; Figure 4.8). This was not an additive action, but synergistic.

This demonstrates that while a constituent may be more concentrated than another in a PEO, it does not necessarily mean that it is responsible for the efficacy of the PEO.

With myrtle, 2 constituents working in combination provided greater antitrypanosomal activity. However, the remaining constituents of myrtle are found in low concentrations, which if used in these experiments would prove very difficult to accurately measure. One example of this would be linalool which has previously been demonstrated to inhibit trypanosome growth (Hoet *et al*. 2006) and is found in a small quantity (3.67%; Appendix 3) in myrtle oil. Such a concentration of constituent would prove very difficult to achieve in the well of a 96 well microtitre plate.

Observation of the experimental microtitre plate wells, using a light microscope, 2 h after the addition of PEOs to the parasites suggested the commencement of epimastigote killing, with epimastigotes in culture beginning to lose motility, starting to condense and become spherical. An experiment was designed and followed to quantify this observation. After 2 h of incubation, viable epimastigotes were enumerated. When incubated with PEOs at their pre-determined MICs, epimastigotes treated with 9 of the 12 PEOs were found reduce viability to 0% (Figure 4.9). Oils of lavender, marjoram and myrtle did not kill all the epimastigotes, but rather 99.3 ± 0.8%, 99.8 ± 0.4% and 99.0 ± 1.1% respectively. The reduction in the activity of the oils was slight, found to be insignificant ($P > 0.05$) for both lavender and marjoram oils and only just insignificant for myrtle oil ($P = 0.0502$).

The rapid action of the PEOs investigated could have some bearing on the potential application of any of the PEOs as novel antitrypanosomal drugs. The faster acting a PEO can be, the greater the chance it has of limiting infection. As the most common route of infection for *T. cruzi* is through entry of epimastigotes / metacyclic trypomastigotes at the mucosal membrane near the eye, nose or mouth, a possible use of PEOs are topical application to the surrounding area of a Reduviid bug bite. The epimastigotes / metacyclic trypomastigotes are excreted in the urine and faeces of the bug as it takes a blood meal from its chosen host. The bite wound is accompanied by pruritus (itch), shows similar symptoms of histamine-induced inflammation within the wound, causing reddening of the skin, plasma extravasation, the development of a weal (tissue oedema) and a flare (wider spread erythema). The action of scratching the irritated bite allows the epimastigotes / metacyclic trypomastigotes in the urine

and faeces to enter through the skin into the wound. Topical application of oils directly to the bite and its surrounding area may help clear the skin of potentially infective *T. cruzi* epimastigotes / metacyclic trypomastigotes and thus reduce the risk of infection. Current drugs used to treat Chagas' disease are only effective during the acute phase of infection. The acute phase is usually asymptomatic making effective early treatment difficult. Therefore, the prevention of entry to viable epimastigotes / metacyclic trypomastigotes may help in the overall treatment and management of *T. cruzi* infection. Most acute cases resolve over a period of 2 to 3 months into an asymptomatic chronic stage which at the moment is untreatable, with only the management of the clinical manifestations of the disease being possible.

A secondary benefit of some essential oils is that they may actually reduce the irritation accompanied with the bite, helping to alleviate the pruritus and thus reducing the compulsion to scratch the affected area. Lavender oil has been used as an alternative medicine for the treatment of insect bites and burns as it is able to reduce pruritus (Tisserand & Balacs 1995). This, however, has no scientific corroboration. Although, TTO has been used traditionally for the relief of discomfort caused by the symptoms of insect bites and has been scientifically tested. This oil has been demonstrated to reduce histamine induced inflammation in human volunteers (Koh *et al*. 2002) and also reduces histamine induced oedema in murine ears (Brand *et al*. 2002). As histamine is released to cause the swelling, redness and itchiness associated with insect bites, it is possible that the ability of the oil and its major constituent terpinen-4-ol, to reduce histamine induced inflammation may be of benefit in controlling the discomfort of insect bites.

Historically, many plant oils and extracts, such as TTO, myrrh and clove, have been used as topical antiseptics, or have been reported to have antimicrobial properties (Hoffman 1987; Lawless 1995). It is important to investigate scientifically those plants which have been used in traditional medicines as potential sources of novel antimicrobial compounds (Mitscher *et al*. 1987). Topical application of essential oils should not be problematic as many are currently used in that manner today in the practice of aromatherapy. When used on the skin for aromatherapy, oils are usually

mixed with inert carrier oils such as almond oil and can be found in concentrations varying from 1 – 30% (Tisserand & Balacs 1995). This is obviously far greater than the concentrations that have been used in the project experiments.

It is clear that there is potential for further investigation in the antitrypanosomal properties of these oils, especially myrtle oil with its low MIC of 0.00125% (12.5 μg ml^{-1}), IC_{90} of 0.0003% (3 μg ml^{-1}) and IC_{50} of 0.0002% (2 μg ml^{-1}) which is within the range of benzidazole (IC_{50} = 2.82 – 16.9 μM [0.73 – 4.4 μg ml^{-1}]). The very properties of PEOs that lend them for use in aromatherapy can be of benefit for any future applications as novel treatments for Reduviid bug bites by having a dual purpose. That is i) relieving the discomfort of the bite itself and thus preventing entry of the parasite into the wound by scratching and ii) killing the epimastigotes on the skin before they have a chance to infect the host.

CHAPTER 5

The effect of blueberry extracts on *Giardia duodenalis* viability and spontaneous excystation of *Cryptosporidium parvum* oocysts, *in vitro*.

5:1 ABSTRACT

The protozoan parasites *G. duodenalis* and *C. parvum* are common causes of diarrhoea, worldwide. Effective drug treatment is available for *G. duodenalis*, but with anecdotal evidence of resistance or reduced compliance. There is no effective specific chemotherapeutic intervention for *Cryptosporidium.* Recently, there has been renewed interest in the antimicrobial properties of berries and their phenolic compounds but little work has been done on their antiparasitic actions. The effect of various preparations of blueberry (*Vaccinium myrtillus*) extract on *G. duodenalis* trophozoites and *C. parvum* oocysts were investigated. Pressed blueberry extract, a polyphenolic-rich blueberry extract and a commercially produced blueberry drink (Bouvrage) all demonstrated antigiardial activity. The polyphenol-rich blueberry extract reduced trophozoite viability in a dose dependent manner. At 167 μg ml^{-1}, this extract performed as well as all dilutions of pressed blueberry extract and the Bouvrage beverage (9.6 ± 2.8% live trophozoites remaining after 24 h incubation). The lowest dilution of blueberry extract tested (12.5% v/v) contained >167 μg ml^{-1} of polyphenolic compounds suggesting that polyphenols are responsible for the reduced survival of *G. duodenalis* trophozoites. The pressed blueberry extract, Bouvrage beverage and the polyphenolic-rich blueberry extract increased the spontaneous excystation of *C. parvum* oocysts at 37ºC, compared to controls, but only at a dilution of 50% Bouvrage beverage, equivalent to 213 μg ml^{-1} gallic acid equivalents in the polyphenolic-rich blueberry extract. Above this level, spontaneous excystation is decreased. The conclusion is that water soluble extracts of blueberries can kill *G. duodenalis* trophozoites and modify the morphology of *G. duodenalis* and *C. parvum*.

5:2 INTRODUCTION

The protozoan parasites, *Giardia duodenalis* (syn. *G. lamblia*, *G. intestinalis*) and *C. parvum* are common causes of diarrhoea, worldwide. Effective drug treatment is available for *G. duodenalis*, but with anecdotal evidence of resistance. Oral metronidazole is the drug of choice, but side effects such as nausea, headaches and an unpalatable strong metallic taste reduce patient compliance. Trophozoites from infective cysts infect the duodenum, attaching onto enterocytes through hydrodynamic forces and microtubule contraction of their ventral 'sucking' disc (Kulda & Nohyhová 1995; Holberton 1974; Céu Sousa *et al*. 2001) and by lectins localised on the trophozoite plasma membrane (Céu Sousa *et al*. 2001; Farthing, Pereira and Keusch 1986; Lev *et al*. 1986; Ward *et al*. 1987), which are specific for D-glucosyl and D-mannose residues (Faubert 2000; Farthing, Pereira and Keusch 1986). In poorer countries where giardiasis is endemic, chemotherapy may be minimally effective due to continual re-infection from the environment (Harris *et al*. 2000).

Chemotherapy for cryptosporidiosis is limited, with nitazoxanide being the only drug to be recommended for use with immunocompetent patient's ≥ 1 years of age (US FDA 2004). Of the 19 valid *Cryptosporidium* species and 40 genotypes, 8 species and 5 genotypes are known to infect humans, with *C. parvum* and *C. hominis* accounting for the majority of human infections (Smith 2008; Smith *et al*. 2007; Feltus *et al*. 2006; Leoni *et al*. 2006; Nichols, Campbell and Smith 2006; Caccio *et al*. 2005; Ryan *et al*. 2004; Xiao *et al*. 2004). Of the *Cryptosporidium* species, *C. parvum* is the most readily available commercially and has been the most commonly used species in scientific investigations.

Ingested, infectious *C. parvum* oocysts are exposed to body temperature, the acid environment of the stomach followed by the alkaline environment of the proximal small intestine and trypsin and bile salts. These major host triggers cause the sporozoites within intact infectious oocysts to excyst and initiate the infection of enterocytes (Smith, Nichols and Grimason 2005). A small percentage (<20% depending on oocyst age and isolate) of sporozoites will excyst when incubated at

37ºC over a 24 h period, in the absence of any of the other major host triggers (Smith, Nichols and Grimason 2005). Excystation at elevated (37ºC) temperature in the absence of other major host triggers has been named 'spontaneous excystation'. Interventions in the *Cryptosporidium* life cycle that reduce the ability of sporozoites to infect enterocytes can become potentially effective treatments for cryptosporidiosis. As sporozoites are the first infective, extracellular stages generated in the host, interventions that influence the timing of their release from intact oocysts, including excystation in abnormal sites, can be considered potentially therapeutic. Possible therapeutic benefits of spontaneous excystation include i) release of *C. parvum* (but not *C. muris*) sporozoites in the acidic environment of the stomach leading to their premature lysis and ii) increased excystation in the intestine, leading to a reduction in the excretion of infectious oocysts.

Plants and plant products have been used as traditional remedies for various ailments in numerous countries, with little or no side effects (Jones 1996). Blueberries (*Vaccinium myrtillus*, syn. Blaeberries, Whortleberries, Bilberries) have been used to treat 'fluxes' (florid diarrhoea) historically in the UK. This is demonstrated in the following passage: "…sell us whortle berries, or the vaccinia nigra of Virgil,… …the astringency lies all in the black skin and not in the pulse. Our soldiers eat them for the bloody flux, while encamped at Fort-Angus (Fort Augustus – Ed.)…our men were getting flux from loch [Ness] water…" – Anon Physician, 3rd September 1746 (Anon. 1825). The true causes of such fluxes are poorly understood, but could be due to various enteropathogens (Haydock 2002; Cox 2002) including *Giardia* and *Cryptosporidium*.

Other evidence for the treatment of fluxes using blueberries comes from the study of archaeological material at microscopic level. An archaeobotanical / archaeomedical project investigating the contents of a sealed hospital drain dating from the early 14th century AD (Soutra) in Scotland, UK discovered *Giardia* cysts in human coprolites in the presence of blueberries. Also identified were *Ascaris* and *Trichuris* ova in the presence of common tormentil (*Potentilla erecta*) and wild strawberry (*Fragaria vesca*) (Personal Communications, Dr. Brian Moffat, Director of the Soutra Hospital

Archaeoethnopharmacological Research Project [SHARP], Edinburgh, 2006). The Augustinian canons at that hospital and other sites used these plants as remedies for helminthic infections and blueberries, in particular, for diarrhoea (Webber & Watson 1998).

The antimicrobial properties of plant products have been recognised for numerous years and recently there has been renewed interest in berries and their phenolic compounds such as anthocyanins and ellagitannins (Nohynek *et al*. 2006; Puupponen-Pimiä *et al*. 2005a & 2005b). Polyphenol-rich berry extracts inhibit the growth of enteropathogenic bacteria such as *Salmonella, Escherichia, Staphylococcus, Helicobacter, Bacillus, Clostridium* and *Campylobacter* species and blueberry polyphenolics are bacteriostatic for three *Staphylococcus aureus* strains (Puupponen-Pimiä *et al*. 2005b). The predominant polyphenolic compounds found in blueberries are the flavonoids, especially the anthocyanins (i.e. anthocyanidin glycosides), which are responsible for the deep red or blue colouring of many berries (Häkkinen *et al*. 1999).

Inactivation and short-term immobilisation of viable bacteria or protozoan parasites by berry extracts might be exploited to inhibit their adhesion to and their colonisation of, human enterocytes. Proanthocyanidins found in cranberry juice (*Vaccinium macrocarpon* and in other *Vaccinium* species such as blueberry) have potent bacterial anti-adhesion activity (Puupponen-Pimiä *et al*. 2005b; Howell 2002).

Plant products also possess anti-protozoan effects, but little is known. Garlic extract inhibits *G. duodenalis* trophozoite growth *in vitro*, primarily due to the transformation product (diallyl trisulphide) of its major constituent (allicin) (Lun *et al*. 1994). Treatment of humans with garlic extract can reduce clinical signs and symptoms more rapidly than metronidazole, the drug of choice (Soffar & Mokhtar 1991). Whole garlic extract co-cultured with trophozoites causes internalisation of flagella and ventral disc fragmentation (Harris *et al*. 2000; Anthony, Fyfe and Smith 2005). Thyme oil can also be as effective as metronidazole in killing *G. duodenalis* cysts (Sahebani, Farsangi and Movahed 2004). Only 2 plant extracts have been tested

against *Cryptosporidium* with any success. Garlic extract caused a mild (22.4%) reduction in oocyst output in *C. baileyi* infected chickens and its effect was comparable to 2 commercially available derivatives of an anticoccidial drug (triazinone) (Sreter, Szell and Varga 1999). An extract of pine bark significantly reduced oocyst shedding in *C. parvum* infected, immunosuppressed C57BL/6N mice. However, it failed to decrease parasite colonisation of the intestine (Kim & Healey 2001).

There are no published scientific data available on the anti-protozoan effects of berries and their polyphenolic compounds, despite their proven anti-bacterial effects. Here, we investigate a pressed extract of blueberries, a commercial drink made from the extract and a polyphenolic-rich extract for their ability to influence i) *Giardia duodenalis* trophozoite viability over time, *in vitro* and ii) the percentage spontaneous excystation of *C. parvum* oocysts.

5:3 MATERIALS AND METHODS

5:3.1 Source of parasites

Source of G. duodenalis trophozoites

Trophozoites of the BVM strain of *G. duodenalis* were maintained axenically in flat sided 110 mm x 16 mm culture tubes at 37ºC, in a modified TYI-S-33 medium (Keister 1983) supplemented with 10% (v/v) heat-inactivated foetal bovine serum. Sub-culturing was performed routinely at 72 - 96 h intervals as described in Chapter 2.

Source of oocysts

Purified *C. parvum* oocysts, passaged in calves, were purchased from Bunch Grass Farm (BGF; Lots 06-4 and 06-30), Idaho, USA and were stored in sterile distilled water containing 100µg of Streptomycin ml^{-1} and 100U Penicillin ml^{-1} at 5ºC until used. Oocysts were <3 months old when used.

5:3.2 Experimental treatments of parasites

Pressed blueberry extract and beverage preparation

The extract from pressed Finnish Blueberries (FB) is used to produce the commercial beverage (Bouvrage Blaeberry; BB). BB contains 25% FB. Both were kindly supplied by Ella Drinks Ltd., Scotland, UK. For the *G. duodenalis* experiments, FB and BB were diluted in TYI-S-33 and adjusted to pH 7 with 0.1M NaOH, then passed through a sterile 0.22 µm filter (Sartorius, Germany). For the *Cryptosporidium* experiments, FB and BB were diluted in Hanks' balanced salt solution (HBSS; Gibco, UK) and adjusted to pH 7 with 0.1M NaOH.

Preparation and analysis of polyphenol and anthocyanin contents of blueberry polyphenolic-rich extract

Polyphenolic-rich extracts of Scottish-grown blueberries (PRBE) (*Vaccinium myrtillus*, variety Berkley) were purified by solid phase extraction using the method of Ross, McDougall and Stewart (2007). The polyphenolic and anthocyanin contents of PRBE, FB and BB were determined using a modified Folin-Ciocalteu method and by differential colorimetric methods (Ross, McDougall and Stewart 2007). The

polyphenol content of PRBE was 920 ± 15 μg gallic acid equivalents (GAE) ml^{-1} and the anthocyanin content was measured at 780 μg cyanidin-3-glucoside equivalents (CGE) ml^{-1}. Aliquots of the polyphenolic-rich extract, containing 200 μg phenol content as GAE, were dried using a speed-vac centrifugal evaporator. This was carried out by staff at the Scottish Crop Research Institute (SCRI, Invergowrie, UK). Dried aliquots were reconstituted in TYI-S-33 at a concentration of 2 mg ml^{-1} then passed through a sterile 0.22 μm filter (Sartorius) for the *G. duodenalis* experiments or in HBSS to make 200 μg ml^{-1} stock solutions for the *C. parvum* experiments.

Incubation of G. duodenalis trophozoites with FB, BB and PRBE

After 72 h of culture, trophozoites were harvested by chilling culture vessels in iced water for 20 min. Experiments were preformed in sterile, 96 well microtitre plates covered with plate sealer film and lids. To 100 μl of trophozoite culture (~2.7 x 10^4 trophozoites) was added 200μl of FB diluted in TYI-S-33, to give a final concentration of 50%, 25% or 12.5% v/v in 300 μl. Similarly, 200 μl of BB, diluted in TYI-S-33, to give a final concentration of 50% v/v in 300 μl was added to 100 μl of trophozoite culture (~2.7 x 10^4 trophozoites). The microtitre plates were sealed and incubated for 24 h at 37ºC, after which the trophozoites were enumerated. Final concentrations of 167, 133, 67 and 33 μg ml^{-1} of PRBE (diluted from a 2 mg ml^{-1} stock into a final volume of 100μl of 2.7 x 10^5 trophozoites ml^{-1} and 200 μl TYI-S-33) were used.

The positive control consisted of 2.7 x 10^4 trophozoites in 300 μl TYI-S-33 containing 67 μg ml^{-1} metronidazole (determined by titration as described in Chapter 2) and the negative controls consisted of 2.7 x 10^4 trophozoites in 300 μl TYI-S-33 in the absence of blueberries or metronidazole. All samples were prepared in triplicate.

Incubation of C. parvum oocysts with FB, BB and PRBE

To each 1.5 ml microcentrifuge tube, 100 μl of a 2 x 10^7 ml^{-1} stock suspension of *C. parvum* oocysts were added to give a final concentration of 2 x 10^6 ml^{-1} oocysts suspended in either FB (50% v/v), BB (50% v/v) or PRBE (213 μg and 860 μg ml^{-1}

GAE, equivalent to 50% BB and 50% FB, respectively) diluted in HBSS. Samples were vortexing then incubated for 24 h at 37ºC.

5:3.3 Parasite enumeration and analysis

G. duodenalis cell count assay

Trophozoite enumeration was performed using an improved Neubauer haemocytometer and trophozoite viability determined by Trypan blue inclusion / exclusion, motility and morphology (TBMM) as previously described in Chapter 2

Oocyst analysis

Following incubation, each sample was washed in HBSS, centrifuged (12,500 x ***g*** for 30 sec), the supernatant aspirated to waste and the pellet resuspended in HBSS. This was repeated three times and the percentage excystation (as an estimate of spontaneous excystation) was performed as previously described in Chapter 3. The control consisted of 100 µl of *C. parvum* oocyst stock suspension mixed with 900 µl HBSS and incubated for 24 h at 37ºC in the absence of blueberry extract.

Statistical analysis

Results are the means of triplicate counts performed on 2 separate experiments. A Student's 2-tailed, unpaired, t-test was used to determine significance with a *P* value of less than or equal to 0.05 being regarded as significant. Data and statistical analysis was performed using Microsoft Excel® software.

5:4 RESULTS

Polyphenolic content of FP, BB and PRBE

The polyphenolic content of FP, BB and PRBE are presented in Table 5.1. All FB and BB samples contained greater than the GAE content of the most effective concentration (167 µg ml^{-1}) of PRBE in the *G. duodenalis* trophozoite viability experiments.

Table 5.1 Composition of FB and BB.

	BB (100%)	**BB (50%)**	**FB (100%)**	**FB (50%)**	**FB (25%)**	**FB (12.5%)**
Polyphenol Content (µg GAE ml^{-1})	426 ± 12	213	1720 ± 13	860	430	215
Anthocyanin content (µg CGE ml^{-1})	254 ± 9	127	1239 ± 19	619	309	155

Key.
BB = Bouvrage beverage
FB = pressed Finnish blueberries

The polyphenol and anthocyanin contents of the polyphenol-rich blueberry extract (PRBE), the pressed Finnish blueberries (FB) and Bouvrage beverage (BB) were expressed as a gallic acid equivalents (GAE) and cyanidin glucoside equivalents (CGE) respectively.

Survival of G. duodenalis trophozoites in FB, BB and PRBE

Viable (motile and sessile) trophozoites excluded Trypan blue. Motile trophozoites exhibited forward movement during which they rotated around their longitudinal axis displaying both a tumbling movement resembling that of a falling leaf and an up and down movement known as 'skipping'. Sessile (viable) trophozoites excluded Trypan blue and retained their typical pyriform shape and internal morphology (Figure 5.1 A, panels A1 - A3). Dead trophozoites included Trypan blue and often their typical pyriform shape became either slightly misshapen or rounded and swollen (Figure 5.1 A, panels A4 - A9). Also found was evidence of blebbing of the trophozoite surface membrane (Figures 5.1 A, panels A4 and A7) and flagellar truncation (Figure 5.1 A, panel A7).

Incubation of trophozoites in the presence of FB significantly reduced their viability over the 24 h period studied ($P \leq 0.01$) compared to untreated trophozoites (Figure 5.2). BB had the greatest effect on trophozoite survival with only 6.5 ± 1.8% trophozoites remaining viable after 24 h. As the concentration of FB used in BB is 25%, then 50% v/v BB is equivalent to 12.5% v/v FB. All concentrations of FB significantly reduced trophozoite viability (50% = 8.4 ± 2.9%; 25% = 10.5 ± 3.7%; 12.5% = 10.8 ± 3.8%) but not in a dose dependent manner. Interestingly, 50% BB was significantly more effective ($P = 0.037$) than 25% and 12.5% v/v FB.

Trophozoite survival was also inhibited by PRBE, but in a dose dependent manner (Figure 5.3). At a dose of 167 μg ml^{-1}, PRBE was as effective as any dilution of FB tested (167 μg ml^{-1}= 9.6 ± 2.8%; 50% v/v FB = 8.4 ± 2.9%; 25% = 10.5 ± 3.7%; 12.5% = 10.8 ± 3.8%; no significant difference between treatments).

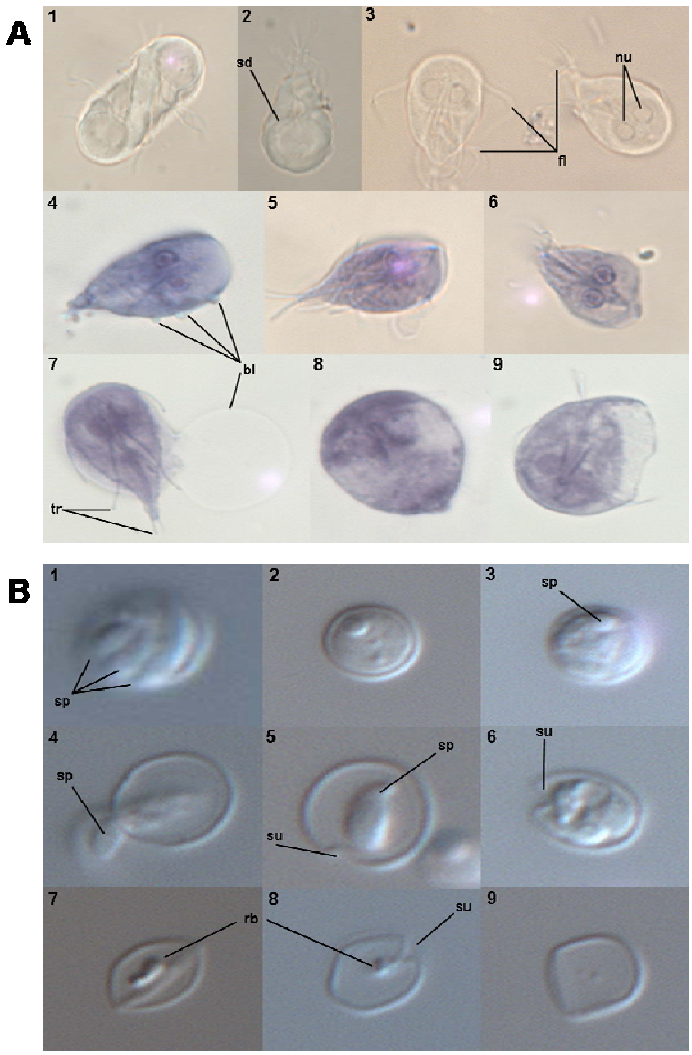


Figure 5.1 A Viable and dead *G. duodenalis* trophozoites exposed to extracts of blueberry.
Figure 5.1 B *C. parvum* spontaneous excystation
Pictures were taken using a ColorView Soft Imaging System MTV-3 camera and analySIS® software. Trophozoites were incubated for 24 h in the presence or absence of FB, BB or PRBE and viable trophozoites were viewed under bright field microscopy (x1250 total magnification). Viable *G. duodenalis* trophozoites are shown in division (panel A1); in ventral view, showing typical pyriform appearance and ventral sucking disc (**sd**) (panel A2); in dorsal view, exhibiting flagella (**fl**) and the 2 characteristic nuclei (**nu**) (panel A3). Dead trophozoites included Trypan blue and often retained their pyriform shape. Some were slightly misshapen (panels A4 -7) or swollen (panels A8 and A9). In some trophozoites, blebbing (**bl**) was evident (panels A4 and A7) and flagella appeared truncated (**tr**) (panel A7). All treatments caused changes in trophozoite morphology and panels A4 – 9 show typical examples of these changes.

Viable *C. parvum* oocysts as seen under Nomarski DIC optics (x1250 total magnification) (panels B1 - 3). Three sporozoites (**sp**) can be seen in panel B1. The unexcysted oocysts in panels B2 and B3 show internalised sporozoites (**sp**). Sporozoites exit through the suture (**su**) in the oocyst wall (panels B4, B5 and B6). Panels B4 and B5 show partially excysted oocysts. Panels B7, B8 and B9 show empty oocysts retaining the residual body (**rb**) and suture (**su**).

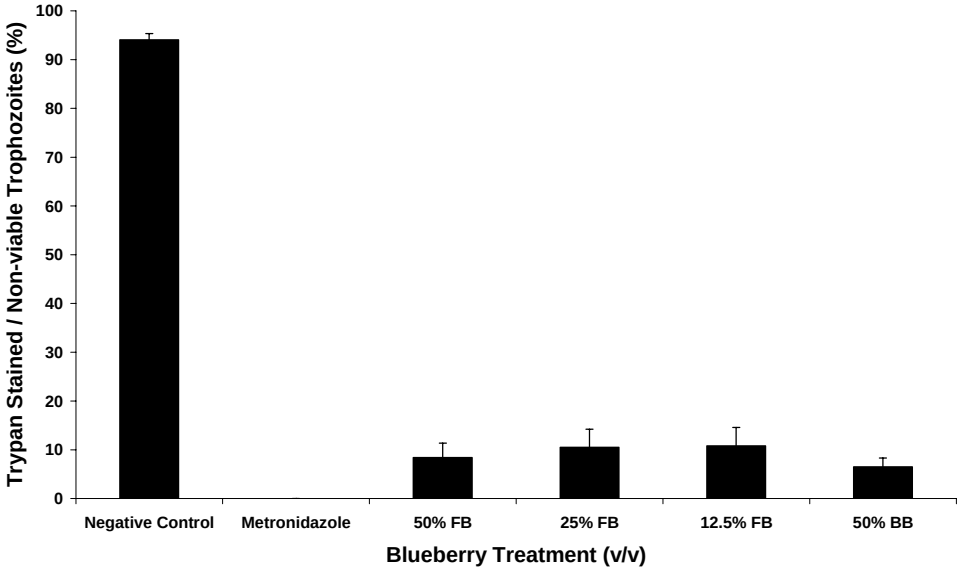


Figure 5.2 Effect of blueberry juice on *G. duodenalis* trophozoite survival *in vitro*.
Trophozoites were incubated for 24 h in the presence or absence of pressed blueberry juice (FB) or the commercially available Bouvrage Blaeberry (BB). Metronidazole (67 μg ml^{-1}) was used as positive control. Both FB and BB significantly reduced the survival of *G. duodenalis* trophozoites when compared to untreated trophozoites ($P \leq 0.01$).

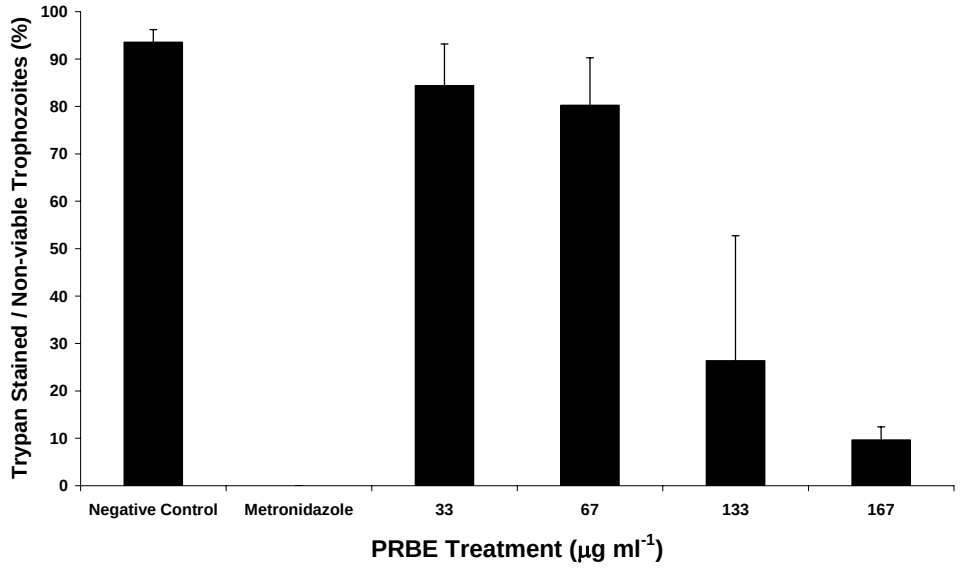


Figure 5.3 Effect of blueberry juice phenolics extract on *G. duodenalis* trophozoite survival *in vitro*.
Trophozoites were incubated for 24 h in the presence or absence of phenolic extract of blueberries (PRBE). Metronidazole (67 μg ml^{-1}) was used as positive control. All concentrations of PRBE significantly reduced the survival of *G. duodenalis* trophozoites when compared to untreated trophozoites ($P \leq 0.01$) but there was no significant difference between the treatments.

Effect of FB, BB and PRBE on the spontaneous excystation of C. parvum oocysts

Typical images of totally excysted (empty), partially excysted and intact oocysts are shown in Figure 5.1 B (panels B1 – B9). Spontaneous excystation, whereby sporozoites within intact oocysts excyst without the classical triggers of acidification and bile, requiring only an increase in temperature and time, was increased in the presence of FB or BB, compared to controls. When *C. parvum* oocysts were incubated for 24 h in the presence of 50% v/v FB or BB, a significant increase ($P \leq 0.01$) in spontaneous excystation was observed, compared to untreated oocysts (Figure 5.4). Incubation in 50% v/v BB produced the greatest level of spontaneous excystation compared to untreated oocysts (40.5 ± 2.4% *vs.* 20 ± 4.3%, $P \leq 0.01$) which was significantly greater ($P \leq 0.01$) than the effect exerted by 50% v/v FB (40.5 ± 2.4% *vs.* 31.3 ± 1.1%).

PRBE at 213 µg and 860 µg ml^{-1} (Figure 5.5) significantly increased spontaneous excystation compared to untreated oocysts (213 µg ml^{-1} = 23.8 ± 3.2%; 860 µg ml^{-1} = 18.0 ± 2.4% *vs.* 10.7 ± 1.9%). As before, incubation in 213 µg ml^{-1} PRBE (50% v/v BB equivalent) produced the greatest level in spontaneous excystation compared to untreated oocysts (23.8 ± 3.2% *vs.* 10.7 ± 1.9%, $P \leq 0.01$) which was significantly greater ($P \leq 0.05$) than the effect exerted by 860 µg ml^{-1} PRBE (50% v/v FB equivalent) (23.8 ± 3.2% *vs.* 18.0 ± 2.4%).

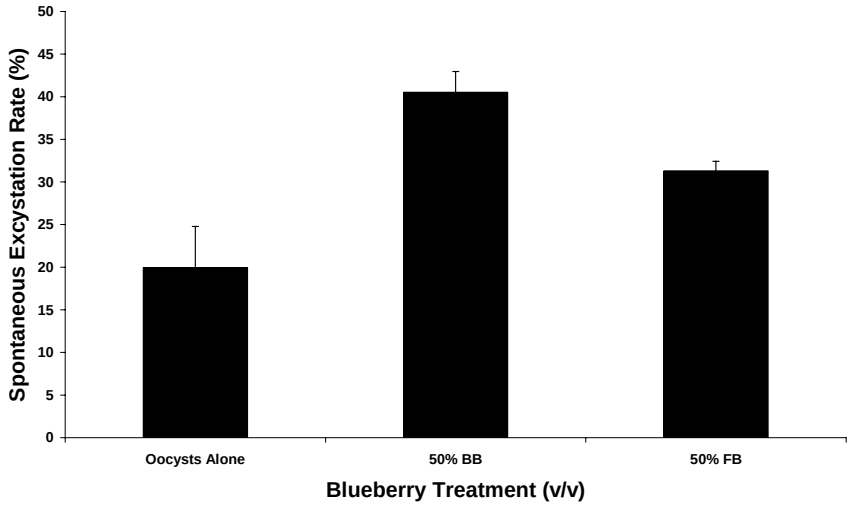


Figure 5.4 Effect of FB and BB on the spontaneous excystation of *C. parvum* oocysts. Oocysts were incubated for 24 h in the presence or absence of pressed blueberry juice (FB) or commercially available Bouvrage Blaeberry (BB) both at 50% concentration (v/v). The results are the means of three counts ± SEs. Both FB and BB significantly increased *C. parvum* oocyst spontaneous excystation when compared to untreated oocysts ($P \leq 0.01$) with BB performing significantly better than FB ($P \leq 0.01$).

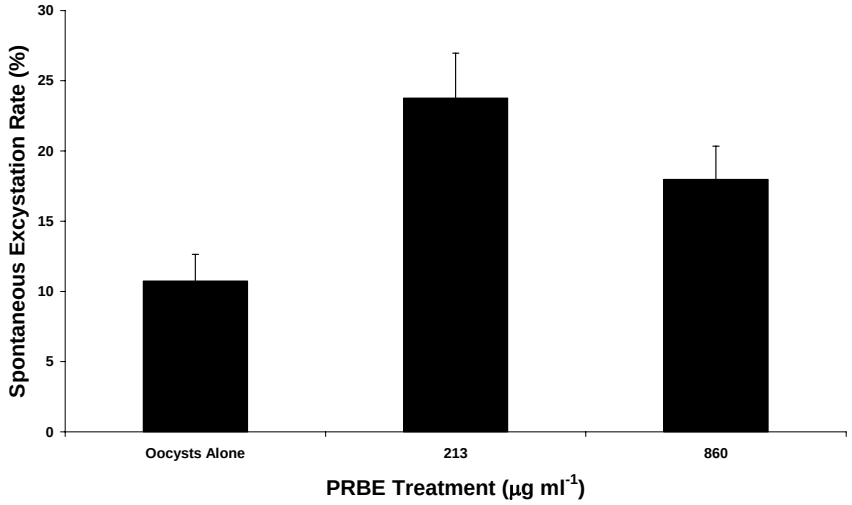


Figure 5.5 Effect of PRBE on the spontaneous excystation of *C. parvum* oocysts. Oocysts were incubated for 24 h in the presence or absence of a polyphenolic rich blueberry extract (PRBE). The results are the means of three counts ± SEs. Both concentrations of PRBE significantly increased *C. parvum* oocyst spontaneous excystation compared to untreated oocysts ($P \leq 0.01$) with 213 μg ml^{-1} performing significantly better than 860 μg ml^{-1} ($P \leq 0.05$).

5:5 DISCUSSION

Here it is shown that extracts of blueberries influence the cell biology of *G. duodenalis* and *C. parvum* over time. Light microscopy was used to identify morphological changes in these parasites. Incubation of *G. duodenalis* trophozoites in extracts of blueberries caused their distortion, swelling, blebbing, flagellar truncation and increased killing, while these extracts increased the rate of spontaneous excystation of *C. parvum* oocysts. As the extracted juice of blueberries is acidic, the pH of FB, BB and the diluents used (TYI-S-33 and HBSS) were adjusted to pH 7 before addition to the parasites to ensure that any effect observed was not due to an acidic environment. Incubation of *G. duodenalis* trophozoites with FB significantly reduced their viability over the 24 h period studied. The commercial beverage (BB) was most effective in killing *G. duodenalis* trophozoites, with only 6.5 ± 1.8% of trophozoites remaining viable after 24 h. Incubation in 50% v/v FB produced similar results (8.4 ± 2.9% trophozoite viability).

There was no significant increase in trophozoite killing between 50%, 25% or 12.5% v/v FB and maximal inhibition occurred at the lowest concentration used. Interestingly, 50% BB (which contains the equivalent of 12.5% v/v FB) was significantly more effective (P = 0.037) than 25% and 12.5% v/v FB in killing trophozoites. This is probably because other constituents in BB (such as 5% raw cane sugar) potentiate the effect of FB. PRBE reduced trophozoite viability in a dose dependent manner. At 167 μg ml^{-1}, PRBE was as effective as all dilutions of FB and BB tested.

Given that 167 μg ml^{-1} of polyphenols killed 90.4 ± 2.8% of *G. duodenalis* trophozoites and that the lowest dilution of FB (12.5% v/v) contained at least 215 μg of polyphenolic compounds (Table 5.1), the lack of a FB dose-response can be explained by them containing polyphenols at a concentration in excess of the minimum required to kill ~90% of trophozoites. As PRBE was prepared by solid phase extraction, it lacks organic acids and sugars found in the pressed blueberry extract. This suggests that the polyphenols are responsible for killing trophozoites. Considering that anthocyanins contribute > 70 % of the polyphenol content of FB

and > 80 % of the polyphenol content of PRBE, these compounds are strong candidates to be the active ingredients.

Metronidazole is the drug of choice for giardiasis and, for adults, a standard treatment (up to 2 g daily for between 3 and 7 days) delivers a peak plasma concentration of 40 μg ml^{-1} within 3 h (McEvoy 1995). Ileal and colonic concentrations reach between 70 – 80% of the dose (Houghton *et al*. 1979). *In vitro*, the IC_{90} for metronidazole was calculated as 21.8 μg ml^{-1} (Chapter 2, Table 2.3), with an MIC of 67 μg ml^{-1}. The anthocyanin content (μg CGE ml^{-1}) of BB and PRBE that killed ~90% of trophozoites were similar (50% BB = 127μg ml^{-1}, PRBE = 142 μg ml^{-1}; Figures 5.3 and 5.4), equating, in terms of trophozoite death, to 21.8 μg ml^{-1} metronidazole. As no significant increase in trophozoite killing was observed between 50%, 25% or 12.5% v/v FB (Figure 5.2) and if we adopt the lowest concentration responsible for killing ~90% of trophozoites (12.5% FB = 155μg ml^{-1} CGE) rather than the highest (50% FB = 619μg ml^{-1} CGE), then the anthocyanin levels responsible for killing ~90% of trophozoites are similar (50% BB = 127μg ml^{-1}, 12.5% FB = 155μg ml^{-1}, PRBE = 142 μg ml^{-1}). Unlike metronidazole which has a half-life of approximately 8 h in humans, with 60 – 80% of the drug and its metabolites excreted in the urine and only 6 – 15% excreted in faeces, anthocyanins are retained in the gut longer with almost 85% being excreted in faeces. This should increase the exposure of *G. duodenalis* trophozoites, either attached onto enterocytes or in the lumen, to anthocyanins ingested in aqueous extracts of blueberries. The manufacturer of Bouvrage uses the pressed juice of 80 g of Finnish blueberries in 250 ml of their beverage, therefore the *in vitro* effects that we describe can be found in 40 g of blueberries.

Both FB and BB caused spontaneous excystation of *C. parvum* oocysts at 37ºC, significantly above untreated control levels (31.3 ± 1.1% for 50% v/v FB and 40.5 ± 2.4% for BB *vs.* 20 ± 4.3% for control oocysts) over the 24 h time period. In addition, both concentrations of PRBE tested (213 and 860 μg ml^{-1}) also caused significant increase in spontaneous excystation compared to control oocysts. 50% BB and 213 μg ml^{-1} PRBE have the same GAE content and caused the highest levels of

spontaneous excystation observed (Figures 5.5 and 5.6). Similarly, 50% FB and 860 μg ml^{-1} PRBE have the same GAE content, but the level of spontaneous excystation observed with these was lower than that observed with 50% BB and 213 μg ml^{-1} PRBE (Figures 5.5 and 5.6). In our experiments, this is suggestive that there is a level of polyphenolics which maximise spontaneous excystation in *C. parvum* oocysts (50% BB and 213 μg ml^{-1} PRBE) above which (50% FB and 860 μg ml^{-1} PRBE) spontaneous excystation is decreased. Experiments to investigate this phenomenon further should be performed as should others to generate dose response data for this phenomenon.

As FB, BB and PRBE increase spontaneous excystation significantly, they could be considered for use as oral supplements to reduce the excretion of infectious oocysts in human and non-human cases of *C. parvum* cryptosporidiosis.

Blueberries are rich in polyphenolic compounds such as the flavonoids, of which the anthocyanins (anthocyanidin glycosides) are the predominant group. Anthocyanins can make up >80 % of the total polyphenolic content of blueberry and can reach concentrations of 2 – 5 g kg^{-1} fresh weight, in this fruit (Puupponen-Pimiä *et al*. 2005b). Anthocyanins have antibacterial properties with bacteriostatic effects due to their anti-adhesion properties in *Escherichia coli* (Puupponen-Pimiä *et al*. 2005b). Anthocyanins from cranberry (*Vaccinium macrocarpon*) juice possess anti-adhesion properties as demonstrated by inhibition of *E. coli* adherence to uroepithelium and multiplication *in vitro* (Ofek *et al*. 1991; Foo *et al* 2000; Reid *et al*. 2001). Whole cranberry juice inhibited adhesion of *E. coli* by type 1 fimbriae (mannose specific) in the yeast aggregation assay, to Chinese hamster ovary cells and murine adrenal cells *in vitro*. Cranberry juice also inhibited adhesion and agglutination of P fimbriated *E. coli*, a α-Gal(1→4)β-Gal specific lectin (Zafriri *et al*. 1989).

Blueberry anthocyanins may also reduce *G. duodenalis* trophozoite and *C. parvum* sporozoite attachment to enterocytes in a similar lectin-specific manner. Selective colonisation of enterocytes of the proximal small intestine may be mediated by lectins localised on the trophozoite plasma membrane (Céu Sousa *et al*. 2001;

Farthing, Pereira and Keusch 1986; Lev *et al*. 1986; Ward *et al*. 1987), which are specific for D-glucosyl and D-mannose residues (Faubert 2000; Farthing, Pereira and Keusch 1986). A role for *C. parvum* sporozoite D-galactose/N-acetyl-D-galactosamine (Gal/GalNAc) surface lectin in adherence to intestinal mucin has been suggested (Smith, Nichols and Grimason 2005). Adherence to a substrate is a key event for intestinal pathogens, as it enables an organism to maintain its station against the flow of food. For *Entamoeba histolytica* trophozoite adherence, attachment to colonic mucus is a key event, facilitated through interaction between trophozoite-derived Gal/GalNAc surface lectin and host glycoconjugates (Huston 2004).

Interference with lectin mediated host-parasite attachment, reducing the ability of protozoan enteropathogens to attach onto enterocytes and increasing the likelihood of them being flushed through the gut, could be a further mode of action of FB, BB and PRBE. Blueberry anthocyanins consist of five anthocyanidin aglycones linked to glucosyl, galactosyl and arabinosyl sugar (Macheix, Fleuriet and Billot 1990) and further work should focus on the specific constituents which interfere with the life cycle phenomena (trophozoite viability, lectin mediated enterocyte attachment) described here.

Blueberries may be potentially useful for treating giardiasis. Anthocyanins are the major polyphenol constituent of blueberries and appear to be prime candidates for the anti-giardial effects we report. Although anthocyanins have low serum bioavailability in humans, they have considerable longevity in the gastrointestinal tract and are known to reach the colon largely undegraded (up to 85% of the initial dose) either following their consumption (Kahle *et al*. 2006) or following *in vitro* simulation of human digestion (McDougall *et al*. 2005).

This study has shown that water soluble extracts of blueberries can kill *G. duodenalis* trophozoites *in vitro* and that this phenomenon is most likely due to polyphenolic compounds. Furthermore, water soluble extracts of blueberries increase the spontaneous excystation of *C. parvum* oocysts, but the data indicate that when a

certain concentration is exceeded (>213 μg ml^{-1}GAE), spontaneous excystation decreases. It is suggested that there are 2 possible modes of action for the effects of water soluble extracts of blueberries on enteropathogenic protozoa, namely, i) modification of parasite morphology and life cycle and ii) reduction / inhibition of lectin-mediated attachment to enterocytes. Thus, the historical use of blueberries as an antidiarrhoeal treatment may have further basis in fact.

This chapter was based upon data successfully published in the Journal of Methods in Phytochemistry and Natural Products (reproduced with permission):

Anthony, J.P**.,** Fyfe, L.**,** Stewart, D., McDougall, G. and Smith, H. (2007). The effect of blueberry extracts on *Giardia duodenalis* viability and spontaneous excystation of *Cryptosporidium parvum* oocysts *in vitro*. *Journal of Methods in Phytochemistry and Natural Products*. Vol 42. Issue 4, pp. 399-348.

Blueberry PRBE extract was prepared by Drs. G. McDougall and D. Stewart of the SCRI. Anthocyanin and polyphenol content analysis was also carried out at that establishment.

CHAPTER 6

The effect of polyphenol-rich fruit extracts on *Giardia duodenalis* viability.

6:1 ABSTRACT

Current conventional treatment for *G. duodenalis* infection of humans appears to be effective, albeit with anecdotal evidence of drug resistance occurring and reduced patient compliances due to side effects. Alternatives to metronidazole are being sought and various polyphenol-rich fruit extracts (PRFEs) covering 11 fruits and 5 different families were investigated for antigiardial activity. Extracts from strawberry, blackberry, cloudberry and artic bramble from the Family Rosaceae were able to reduce trophozoite viability by 100%. Of these 4 PRFEs, cloudberry was found to have the greatest antigiardial activity being able to reduce viability at concentrations comparable to the reference drug metronidazole (67 μg ml^{-1}). This activity was not thought to be due to the biodegradation of ellagitannins (found in high quantities within the Rosaceae family) to ellagic acid. The use of cloudberries as a treatment for giardiasis warrants further examination.

6:2 INTRODUCTION

It has been estimated that ~280 million people are at risk from giardiasis worldwide (Lane & Lloyd 2002; Olson, Ceri and Morck 2000). This flagellated protozoan can infect a wide range of mammals with 25 – 100 cysts causing infection in humans (Rendtorff 1954; Smith *et al*. 2007). It is one of the most common waterborne causes of parasite infection world wide (Karanis, Kourenti and Smith 2007). After ingestion the cyst is triggered to excyst following exposure to gastric acids and the alkalinity of the small intestine, the trophozoite excysts from the cyst. The trophozoites colonize the proximal surface of the small intestine and rapidly reproduce by binary fission. Most infections (60%) are asymptomatic with cysts being passed in the faeces of the host to continue the infectious cycle (Ortega & Adam 1997). In other cases, symptomatic infections will present 1 – 2 weeks after cyst ingestion. During the acute phase of symptomatic infection there is a sudden onset of explosive, watery, foul smelling diarrhoea, associated with abdominal distension and an increase in foul flatulence that may last for up to 6 weeks. However, in the immunocompromised and in some immunocompetent hosts, the acute symptoms may last for several months, causing weight loss, malabsorption and steatorrhoea.

Conventional chemotherapy for *G. duodenalis* infection involves the use of the 5-nitroimidazoles, metronidazole and tinidazole. Drug resistance has been shown in the laboratory and there is anecdotal evidence of drug resistance *in vivo* (Upcroft & Upcroft 1993). Oral metronidazole, however, is still the drug of choice, but side effects such as nausea, headaches and an unpalatable strong metallic taste reduce patient compliance.

The plant world can be a source for novel chemotherapeutics with 25% of prescription drugs currently derived from plants (Editorial 1994). Polyphenols from plants have previously been examined for their antiparasitical activity and their antimicrobial with some success (Anthony *et al*. 2007; Kolodziej, Radtke and Kiderlen 2007; Kolodziej & Kiderlen 2006; Mendonca-Filho *et al*. 2004; Radtke *et al*. 2003; Kolodziej *et al*. 2001a & 2001b; Puupponen-Pimiä *et al*. 2005b; Sotohy, Müller and Ismail 1995).

Flavonoids and phenolic acids form the building blocks for polymeric tannins, which can be classified into hydrolysable and condensed tannins. Hydrolysable tannins are glucose esters of gallic and ellagic acids with condensed tannins having a flavonoid core as a basic structure. Fruits, especially of the Family Rosaceae, Genus *Rubus*, are rich in hydrolysable tannins called ellagitannins (Puupponen-Pimiä *et al*. 2005a). These ellagitannins have demonstrated wide ranging antibacterial effects against both gram-negative and gram-postive organisms (Rauha *et al*. 2000) with gallic acids causing the permeabilisation of the *Salmonella* outer membrane, but not anthocyanins (Nohynek *et al*. 2006). Other mechanisms of action include the interference of bacterial adhesion through the interference of lectin binding and mannose specific adhesion of *E. coli* bacteria by anthocyanins (Foo *et al*. 2000; Reid *et al*. 2001; Ofek *et al*. 1991; Zafriri *et al*. 1989).

As attachment to enterocytes is a key event in giardiasis, allowing the trophozoites to maintain position within the small intestine, any interference with attachment could lead to premature transit to the colon and their death. Attachment by *Giardia* is mediated through hydrodynamic forces of the ventral disc and lectin binding to D-glucosyl and D-mannose residues (Céu Sousa *et al*. 2001; Faubert 2000; Farthing, Pereira and Keusch 1986; Lev *et al*. 1986; Ward *et al*. 1987).

As the results of Chapter 5 demonstrated that the polyphenol-rich extract of a fruit (blueberry) has antigiardial activity, possibly through anthocyanin activity, other fruit extracts were examined for giardiacidal properties. PRFEs of 11 fruits from 5 different Families, gifted by the SCRI were investigated for their ability to influence *G. duodenalis* trophozoite viability over time, *in vitro*.

6:3 MATERIALS AND METHODS

6:3.1 Parasite culture

Source of G. duodenalis trophozoites

Trophozoites of the BVM strain of *G. duodenalis* were maintained axenically in flat sided 110 mm x 16 mm culture tubes at 37ºC, in a modified TYI-S-33 medium (Appendix 4) supplemented with 10% (v/v) heat-inactivated foetal bovine serum. Sub-culturing was performed routinely at 72 – 96 h intervals as described in Chapter 2.

Preparation and analysis of polyphenol contents of PRFEs

PRFEs of 11 different soft fruits (Table 6.1) were purified by solid phase extraction using the method of Ross, McDougall and Stewart (2007) at SCRI. The polyphenolic and anthocyanin contents of PRFEs were determined as described in Chapter 5. Dried aliquots were reconstituted in 1 ml TYI-S-33 at a concentration of 2 mg ml^{-1} then passed through a sterile 0.22 μm filter (Sartorius).

Table 6.1 Soft fruits used to create polyphenol-rich extracts.

FAMILY	BOTANICAL NAME	VULGAR NAME
Elaeagnaceae	*Hippophae rhamnoides*	Sea Buckthorn
Ericaceae	*Vaccinium myrtillus*	Blueberry/Bilberry
	Vaccinium vitis-idaea L.	Lignonberry/Cowberry
Grossulariaceae	*Ribes nigrum*	Blackcurrant
Punicaceae	*Punica granatum* L.	Pomegranate
Rosaceae	*Fragaria vesca*	Strawberry
	Rubus arcticus	Artic Bramble
	Rubus chamaemorus	Cloudberry
	Rubus fruticosus	Blackberry
	Rubus idaeus	Raspberry
	Sorbus aucuparia	Rowan

Preparation of ellagic acid

Following the titration selected of PRFEs, a metabolite of ellagitannin which is known to exist in high quantities in the Rosaceae Family of plants, was investigated for its antigiardial activity. This compound (ellagic acid) was found to be highly insoluble in water and required to be diluted in absolute ethanol and water (50% v/v)

before any dilutions in TYI-S-33 could be made at a stock concentration of 2 mg ml^{-1}. This was further diluted in TYI-S-33 medium to a concentration of 1 mg ml^{-1} to reduce the volume of ethanol in the microtitre plate wells in the presence of *Giardia* trophozoites. The highest final concentration of ethanol in the well corresponding to 133.4 µg ml^{-1} ellagic acid was calculated to be 13%. Because of this, an ethanol control was included. Dilutions of ellagic acid started at 133.4 µg ml^{-1}, which corresponded to one dilution above the MIC of cloudberry extract and titrated to 33 µg ml^{-1}. Ethanol concentrations corresponding to that found in ellagic acid solution (13, 6.5 and 3.25%) were also incubated with *Giardia* trophozoites.

6:3.2 Experimental treatments of parasites

Incubation of G. duodenalis trophozoites with PRFE

After 72 h of culture, trophozoites were harvested by chilling culture vessels in iced water for 20 min. Experiments were preformed in sterile, 96 well microtitre plates covered with plate sealer film and lids. Final concentrations of 333.5 µg ml^{-1} of PRFE (diluted from a 2 mg ml^{-1} stock into a final volume of 150 µl of 2.7 x 10^5 trophozoites ml^{-1} and 150 µl TYI-S-33) were initially used. Where 100% killing of trophozoites was observed, the PRFEs responsible were further titrated to concentrations of 266.8, 133.4 and 67 µg ml^{-1}. The microtitre plates were sealed and incubated for 24 h at 37ºC, after which the trophozoites were enumerated.

The positive control consisted of 2.7 x 10^4 trophozoites in 300 µl TYI-S-33 containing 67 µg ml^{-1} metronidazole (see below) and the negative controls consisted of 2.7 x 10^4 trophozoites in 300 µl TYI-S-33 in the absence of PRFE or metronidazole. All samples were prepared in triplicate.

Incubation of G. duodenalis trophozoites with metronidazole

The minimum concentration of metronidazole that killed 100% of trophozoites *in vitro* was previously determined to be 67 µg ml^{-1} (20 µg per well; Chapter 2). This concentration was used in all positive controls.

6:3.3 Parasite culture / experimental analysis

G. duodenalis cell count assay

Trophozoite enumeration was performed using an improved Neubauer haemocytometer and trophozoite viability determined by Trypan blue inclusion / exclusion with motility and morphology (TBMM) as described in Chapter 2.

Statistical analysis

In this series of experiments, *Giardia* trophozoites from each microtitre well from 2 experiments were counted providing a total of 6 enumerations for each PRFE concentration tested. The means and standard deviations of these enumerations were used in formulating graphs and tables, with a Student's 2-tailed, unpaired, t-test used to determine significance with a *P* value of less than or equal to 0.05 being regarded as significant. Data and statistical analysis was performed using Microsoft Excel® software.

6:4 RESULTS

Incubation of G. duodenalis trophozoites with PRFEs

Screening of PRFEs

All 11 PRFEs significantly reduced the viability of *G. duodenalis* trophozoites at a concentration of 167 μg ml^{-1} (Figure 6.1, $P > 0.05$). Only 4 of the 11 investigated were able to reduce trophozoite viability by 100%. These were strawberry, blackberry, cloudberry and artic bramble. Interestingly these 4 fruits are all from the same scientific Family (Rosaceae), however, while both rowan and raspberry are from the same family, these failed to completely reduce trophozoite viability. Raspberry extract killed 11.9 ± 4.7% of the trophozoites while rowan extract was considerably more effective, inducing a 83.3 ± 14.3% reduction in trophozoite viability. However, rowan PRFE activity was not significantly different from that of pomegranate and blueberry (86.2 ± 10.9 and 90.4 ± 2.8% respectively; $P < 0.01$) and was significantly lower than the activities of strawberry, blackberry, cloudberry and artic bramble.

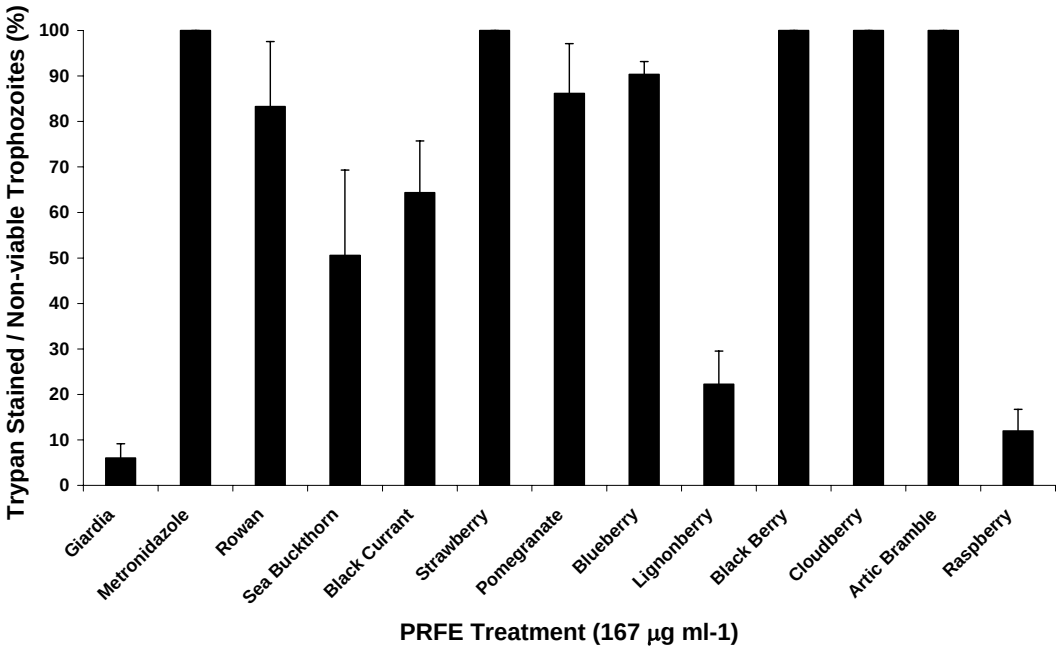


Figure 6.1 Effect of PRFEs on *G. duodenalis* trophozoite survival *in vitro*.
Trophozoites were incubated for 24 h in the presence or absence of PRFE. Metronidazole (67 μg ml^{-1}) was used as positive control. All berry extracts significantly reduced the survival of *G. duodenalis* trophozoites when compared to untreated trophozoites ($P \leq 0.05$).

Titrations of the most active PRFEs

As extracts of strawberry, blackberry, cloudberry and artic bramble were able to kill 100% of *Giardia* trophozoites at 167 μg ml^{-1}, titrations of these extracts were performed down to the lowest concentration of 33 μg ml^{-1}. A dose dependent reduction in the survival of trophozoites was observed for each extract (Figure 6.2). A polyphenol-rich extract of strawberry demonstrated an MIC of 167 μg ml^{-1} with concentrations below this failing to completely reduce trophozoite viability. Both artic bramble and blackberry extracts had an MIC of 133 μg ml^{-1} with cloudberry demonstrating an MIC of 67 μg ml^{-1}. At a concentration of 33 μg ml^{-1}, cloudberry showed an ablation of its antigiardial activity, being equal in effect as the untreated controls (*Giardia* trophozoites alone = 11.3 ± 8.6% *vs*. cloudberry 33 μg ml^{-1} = 12.6 ± 6.0%, Figure 6.2, $P > 0.05$).

Incubation of trophozoites with ellagic acid

The Family Rosaceae of plants is known to have high levels of ellagitannins and as ellagic acid is known to be a metabolite of ellagitannin, this compound was investigated. When used at a concentration demonstrated by PRFEs to kill 100% of trophozoites (133 μg ml^{-1}), ellagic acid appeared to cause a reduction in trophozoite viability (75.2 ± 4.0%), with further dilutions showing a dose dependent decrease in antigiardial activity (Figure 6.3 A). However, the high concentrations of ethanol calculated to be present in the ellagic acid dilutions demonstrate that ethanol is responsible for the reduction of viability and not ellagic acid (Figure 6.3 B). Interestingly, the addition of ellagic acid helps to increase trophozoite survival, which was reduced by ethanol (Figure 6.3). Ethanol, at a concentration of 13%, killed 100% of trophozoites and ellagic acid, at 133 μg ml^{-1} in the presence of 13% ethanol, killed 75.2 ± 4.0% trophozoites, significantly reducing the ethanol induced antigiardial effects ($P < 0.01$).

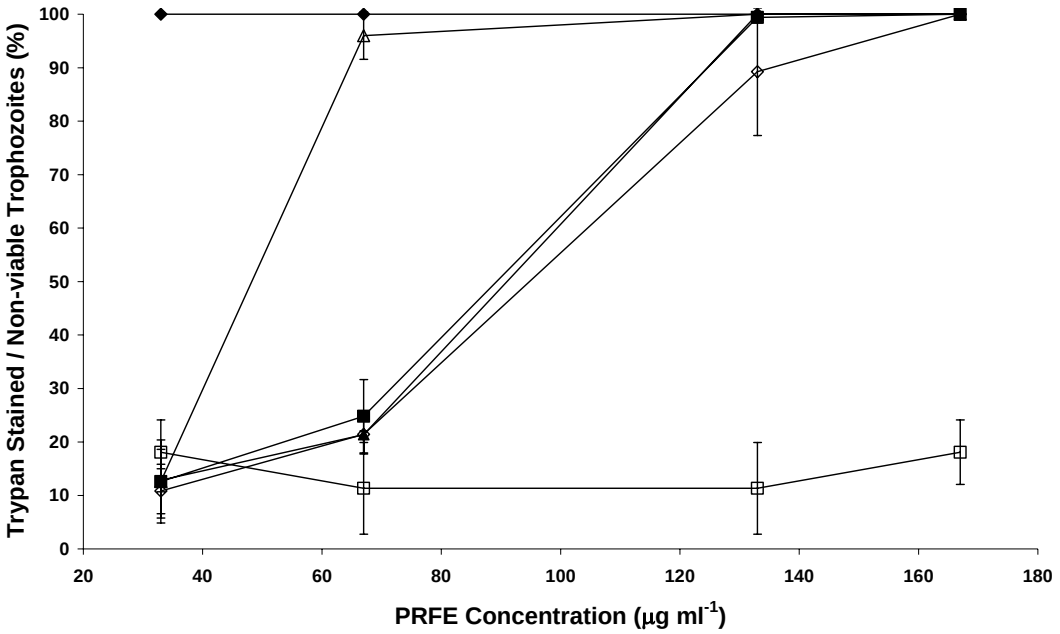


Figure 6.2 Effect of 4 berry phenolic extracts on *G. duodenalis* trophozoite survival *in vitro*.

Trophozoites were incubated for 24 h in the presence or absence of 4 different PRFEs at different concentrations (33, 67, 133 and 167 μg ml^{-1}). Untreated trophozoites (□) and metronidazole 67 μg ml^{-1} treated trophozoites (◆) were used as controls. Strawberry (◇), blackberry (■), cloudberry (△) and artic bramble (▲) caused a dose dependent reduction in trophozoite viability.

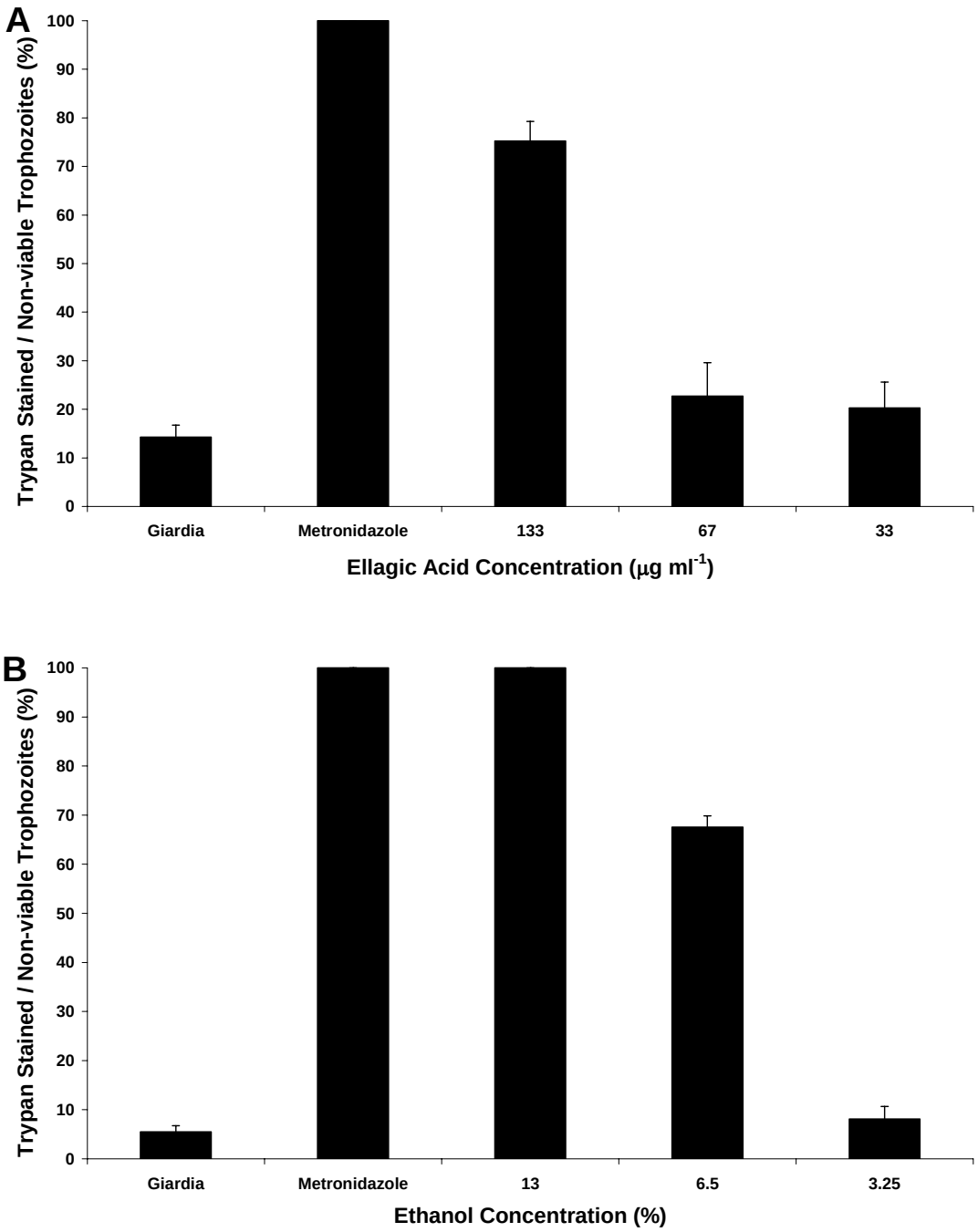


Figure 6.3 Effect of ellagic acid and ethanol on *G. duodenalis* trophozoite survival *in vitro*.
Trophozoites were incubated for 24 h in the presence or absence of ellagic acid (**A**) or ethanol (**B**) at different concentrations (133.4, 67 and 33 µg ml^{-1} for ellagic acid and 13, 6.5 and 3.25% for ethanol). Metronidazole (67 µg ml^{-1}) was used as positive control. Ethanol was used at concentrations corresponding to that found in the ellagic acid dilutions.

6:5 DISCUSSION

It was demonstrated that PRFEs influence the viability of *G. duodenalis* over a 24 h incubation. Four water soluble PRFEs demonstrated high activity against trophozoites of *G. duodenalis in vitro* when compared with the remaining PRFEs. These PRFEs were strawberry, blackberry, cloudberry and artic bramble and represent 4 out of 6 members of the Family Rosaceae that were tested. All 4 PRFEs were able to reduce trophozoite viability by 100% at a concentration of 167 μg ml^{-1} and showed a dose dependent reduction of viability. Cloudberry extract was the most active of all the PRFEs, reducing significantly trophozoite viability at a concentration of 67 μg ml^{-1} ($P > 0.05$).

As PRFEs were prepared by solid phase extraction, they lack organic acids and sugars found in the pressed fruit extracts. This would mean that the remaining phenolic compounds, such as flavonoids, lignans, stilbenes and polymeric tannins (condensed and hydrolysable), are responsible for any giardiacidal effects. The antiparasitical activity of plant phenols has been demonstrated by others. Experiments by Kolodziej *et al*. (2001) provided evidence for antileishmanial activity of proanthocyanidins, condensed tannins which have a flavonoid core as their basic structure. The activities of these phenols were not directly against the parasite, as *L. donovani* promastigotes *in vitro* survived incubation with these compounds. The intracellular survival of amastigotes, however, was significantly inhibited, with activities reported to be lower than that of the drug, Pentostam®. This indicated that the proanthocyanidins were responsible for the activation of intracellular killing mechanisms of infected macrophages.

The antimicrobial properties of the purified phenolic compounds present in many plant foods have been well documented (Chung, Wei and Johnson 1998). However, the complex mixture of phenolics to be found in berry extracts have not been extensively examined until recently. Puupponen-Pimiä *et al*. (2005a) demonstrated that phenolic berry extracts have wide ranging antimicrobial effects by inhibiting their growth, with a phenolic extract of cloudberry possessing the strongest antimicrobial activity, followed by raspberry and strawberry. Rauha *et al*. (2000) also studied antimicrobial effects of some berry extracts against food poisoning bacteria

(*Aspergillus niger*, *Bacillus subtilis*, *Candida albicans*, *Escherichia coli*, *Micrococcus luteus*, *Pseudomonas aeruginosa*, *Saccharomyces cerevisiae*, *Staphylococcus aureus* and *Staphylococcus epidermidis*). They found that the widest bactericidal activity was also expressed by berries belonging to the genus *Rubus* (cloudberry and raspberry) which are rich in the hydrolysable tannins known as ellagitannins, formed from the glucose esters of gallic and ellagic acid.

Nohynek *et al*. (2006) demonstrated that the outer membrane (OM) of *Salmonella typhimurium* and *S. infantis* was disintegrated by phenolic extracts of cloudberries and raspberries. It was believed that this activity occurred through the chelation of divalent cations from the OM as the addition of $MgCl_2$ inhibited the destruction of the OM by these berry phenolics. Further examination discovered that this permeabilisation or disintegration of the OM was not due to the ellagitannin and anthocyanin fractions of raspberry and cloudberry phenolic extracts (Nohynek *et al*. 2006). Also the growth inhibition of *Salmonella* was only partly caused by the berry phenolics and ellagitannins, with most of the inhibition seeming to originate from other compounds, such as organic acids (Puupponen-Pimiä *et al*. 2005a). This would indicate that the ellagitannins from these berries in phenolic extracts are not responsible for their antimicrobial effect on *Salmonella* spp.

Whilst the ellagitannins have been shown not to be responsible for the antimicrobial effects for the disintegration of Gram-negative bacteria OM, they cannot be disregarded for antigiardial effects demonstrated with the ellagitannin rich cloudberry, artic bramble and strawberry PRFEs without some *in vitro* investigation. To this end, purified ellagitannins were sought for use in this project. However, whilst ellagitannins were unavailable, the biodegradation product (ellagic acid) was donated by Dr. McDougall (SCRI) for use in experiments. The breakdown of ellagitannin to ellagic acid by intestinal microflora may cause this phenolic acid to be present in high quantities at sites where *G. duodenalis* trophozoites also occupy. Experiments, however, demonstrated that *Giardia* trophozoites were unaffected by ellagic acid (Figure 6.3). From this it can be suggested that any antigiardial effect of the PRFEs from strawberry, blackberry, cloudberry and arctic bramble may have *in*

vitro, is not due to the degradation products of ellagitannins, i.e. ellagic acid. As the PRFEs are devoid of organic acids and that ellagic acid was found not to affect trophozoite viability, the potential remaining classes of phenolic compounds in PRFEs with antigiardial properties could be the flavonoids, lignans, stilbenes or condensed tannins (Puupponen-Pimiä *et al*. 2005a).

Previous experiments have suggested the possibility that anthocyanins may be responsible for the antigiardial effects of PRFEs (Anthony *et al*. 2007; Chapter 5). This was suggested due to the blueberry PRFE, the whole pressed juice and a commercial drink made from the same juice all containing large amounts of anthocyanins. This was calculated to be 70 – 80% of its polyphenol content being made of anthocyanins. In the current experiments however, the PRFEs which were most antigiardial were from a family known to be particularly high in their ellagitannin content. Also, strawberry and cloudberry phenolics have been shown to cause the immobilisation and death of *S. aureus* and *S. typhimurium* (Nohynek *et al*. 2006). This may have implications in the treatment of diarrhoeal infections where the prevention of adhesion of pathogenic bacteria and possibly protozoan parasites such as *G. duodenalis* to intestinal epithelial cells would be of benefit to the host.

However, it is becoming clear that different classes of polyphenol compounds differ greatly in their bioavailability (Manach *et al*. 2005). Constituents such as anthocyanins, which are abundant in berries (Deighton *et al*. 2000), have low bioavailability and / or poor stability *in vivo* (McDougall *et al*. 2005). A large proportion of the ingested polyphenol dose from berries will not be taken up into the circulation and will pass through the upper gastrointestinal tract (GIT) to the large intestine where they may be biotransformed, or broken down by the indigenous microflora (Aura *et al*. 2005; Gonthier *et al*. 2003). This however, may be of benefit in the treatment of giardiasis. Unlike metronidazole, anthocyanins are retained in the gut longer, with almost 85% being excreted in faeces. This should increase the exposure of *G. duodenalis* trophozoites, either attached onto enterocytes or in the lumen, to polyphenols ingested in aqueous extracts of cloudberries.

However, the antimicrobial activity of phenolic compounds can also depend on the pH of the matrix in which the organism grows. This can vary with different bacterial species and different phenolic compounds. For example, certain phenolic compounds, such as the phenolic acids (the cinnamic and benzoic acids), are unstable at high pH but are stable at low pH, being bactericidal at low pH (< pH 5) and bacteriostatic at higher pH (> pH 6) (Friedman and Jürgens 2000; Puupponen-Pimiä *et al*. 2005a; Wen *et al*. 2003). Thus, when evaluating the antimicrobial efficacy of phenolic compounds either in food as a preservative, or in the human body as chemotherapy, pH is a very important parameter to be considered, especially as a potential use of cloudberry in the treatment of giardiasis could be the oral administration of either the whole fruit, or pressed juice of the fruit. There is some precedence for this, where experiments by Ryan, Wilkinson and Cavanagh (2001) demonstrated that *E. coli* growth was inhibited *in vitro* by a dilution of raspberry juice or cordial, providing some scientific evidence for the common practice of using raspberry cordial as an antidiarrhoeal therapy in livestock and humans in Australia.

This study has shown that PRFEs of various soft fruits can kill *G. duodenalis* trophozoites *in vitro* and that this phenomenon is most likely to be due to polyphenolic compounds. Furthermore, PRFEs of cloudberry, artic bramble, blackberry and strawberry, at concentrations of 167 μg ml^{-1}, completely reduced trophozoite viability. As cloudberry PRFE has activity which is comparable to metronidazole this may indicate the possible use of cloudberries as an antidiarrhoeal treatment induced by giardiasis warrants further examination.

CHAPTER 7

The examination of latrine and cess pit sediments from 2 different localities in Britain and eras of history for protozoan parasites, with a special emphasis on *Cryptosporidium* spp.

7:1 ABSTRACT

Sites of scientific interest from both the Viking and medieval era were examined for the presence of protozoan parasites. Samples from a sealed 14th century AD hospital drain (Soutra Aisle, UK) and from latrine sediments (16 – 22 Coppergate, York, UK) were examined using light and epifluorescent microscopy and nested 18S polymerase chain reaction coupled with restriction fragment length polymorphism (PCR-RFLP) techniques. Both samples were found to contain ova of the helminths *Trichuris* spp. and *Ascaris* spp. Also found were *Cryptosporidium* spp. oocysts, which were visualised using fluorescein isothiocyante conjugated monoclonal antibody (FITCmAb) for *Cryptosporidium* spp. indicating the presence and robustness of the oocyst epitopes which the antibodies are directed against. The 2 step nested 18S PCR-RFLP techniques employed discovered the presence of *Cryptosporidium* spp. DNA in the Soutra samples but not in the York samples, but analysis could not determine the species of the *Cryptosporidium* found. The presence of the *Cryptosporidium* DNA of the same amplicon size in imported topsoil samples from above the archaeological layer, indicate a possible existence of *Cryptosporidium* in the top soil which had leached down into the archaeological layer. Further analysis using other nested 18S PCR-RFLP techniques would be required to ultimately identify the *Cryptosporidium* species found in all DNA positive samples. If these techniques determine that the *Cryptosporidium* DNA present in the imported topsoil samples is different from the archaeological samples, then this will be the first reported finding of *Cryptosporidium* in an European archaeological site.

7:2 INTRODUCTION

The examination of paleo-faecal remains for parasites is becoming routine in archaeological excavations (Araujo & Ferreira 2000). The significance of examining such material is to try to gain an insight into the diseases of the past, diet and possibly the treatment of diseases. Such endeavours have so far been carried out in archaeological sites in North and South America (Horne 1985; Kliks 1990; Ferreira *et al*. 1991; Ferreira, Araujo and Duarte 1993; Schmidt Duszynski and Martin 1992; Araujo & Ferreira 2000; Faulkner *et al*. 2000), Europe (Taylor 1955; Grzywinski 1959; Pike 1968; Herrmann 1988; Jones 1985; Greig 1981; Bouchet 1995; Araujo & Ferreira 2000; Bouchet *et al*. 2002) and Egypt (Ruffer 1910).

Parasite ova from nematodes, cestodes and trematodes are the most commonly found evidence of parasites throughout the world and have been found from a number of different sources including waterlogged deposits, concreted faecal deposits and arid deposits. One such examination has recently discovered trematode and nematode ova in the coprolites of terrestrial vertebrates from the Early Cretaceous Bernissart Iguanodon shaft in Belgium (Poinar & Boucot 2006).

The detection of protozoan parasites in archaeological material, whether it is coprolites, latrine sediments or a mixture of faecal material and soil, is not frequent in palaeoparasitology (the study of parasites in archaeological material) (Goncalves, Araujo and Ferreira 2003). Unlike the ova of helminths, protozoan cysts, such as *Giardia* spp., appear to degrade and quickly decay in the conditions found in archaeological sites (such as high or low moisture levels, cool or hot temperatures). Direct microscopic examination of these parasites is difficult. However, it is possible to detect these parasites using other means. *Giardia* spp. cysts and *Cryptosporidium* spp. oocysts have been identified in human coprolites from ancient Peruvian mummies approximately 4,300 to 1,100 BP (Before Present, i.e. the present year and not BC [Before Christ]) using fluorescence microscopy (Ortega & Bonavia 2003). More recently, intact intestinal protozoan cysts have been found in Early Cretaceous coprolites (Poinar & Boucot 2006).

Of particular interest is a sealed hospital drain / cess pit dating from the early AD (*Anno Domini*) 14th century (Soutra) in Scotland, UK. At this site and other hospital sites, Augustinian canons used common tormentil (*Potentilla erecta*) and wild strawberry (*Fragaria vesca*) plants as remedies for helminthic infections and blueberries, in particular, for the treatment of diarrhoea (Webber & Watson 1998). Currently an archaeobotanical / archaeomedical project (Soutra Hospital Archaeoethnopharmacological Research Project; SHARP) investigating this site is continuing to investigate the medieval use of plants as medicines by the Augustinian canons. This is possible due to the retention of organic material with little decay because of the waterlogged clay soil in which the material is entombed.

This current study was undertaken to attempt to find and identify any intestinal protozoan parasites in hospital drain / cess pit samples from Soutra and also to confirm and identify any other parasites to be found in latrine sediments from a Viking settlement latrine excavated from 16 – 22 Coppergate, York dating from AD 800 – 1000 and to attempt to associate plants with any parasites found. Light and fluorescence microscopy was used for the primary identification of any parasites found and a highly sensitive PCR method was also employed for the identification of *Cryptosporidium* spp. oocysts.

7:3 MATERIALS AND METHODS

7:3.1 Source of archaeological samples

Soutra cess pit samples

Samples of a medieval cess pit (Soutra Aisle, ~17 miles South East of Edinburgh, UK, Ordinance Survey reference: NT 452 584 GB, Figure 7.1) dating from the 14th century AD were obtained from a depth of approximately 1.5 m (Figures 7.2 A and 7.2 B). These samples are of special interest as they are from waste material from a medieval hospital which, in previous archaeological studies, has well preserved organic material, allowing the identification of plants used in medicinal preparations (Dr. Brian Moffat, Personal Communications 2006).

Figure 7.1 Location of Soutra Aisle.

The location of Soutra Aisle within the UK is marked on the map © Crown Copyright 2007 www.ordnancesurvey.co.uk/**getamap** Image produced from Ordnance Survey's Get-a-map service. Image reproduced with permission of Ordnance Survey and Ordnance Survey of Northern Ireland. Scale 1:625,000.

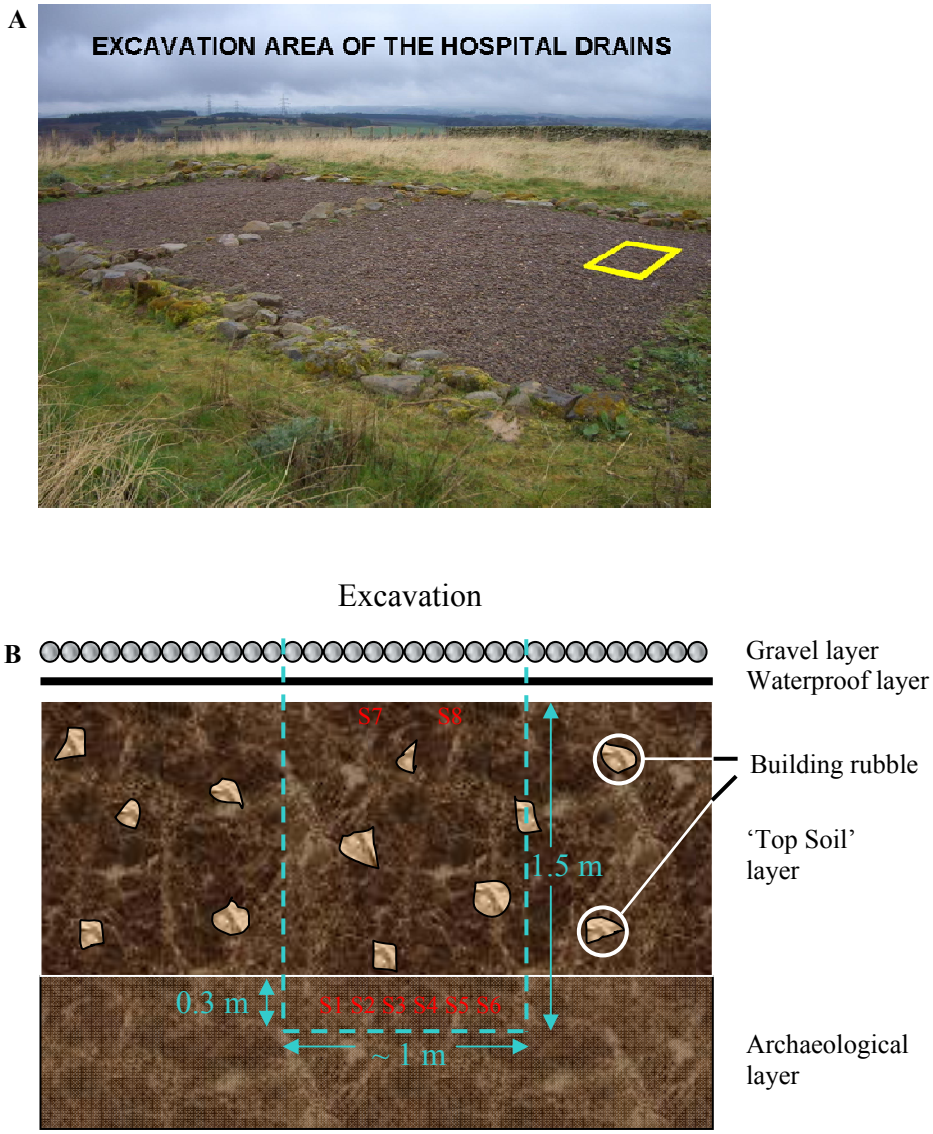
In total, 8 samples of soil were taken from the site with 6 samples from a pristine, untouched layer of the cess pit (sample identifications S1 – S6) and 2 samples from the soil above the archaeological layer (sample identifications S7 & S8). This soil

had been added to the site after previous excavations where the archaeological layer of waterlogged clay was initially exposed and then subsequently protected with this 'top soil' of loam and building rubble mixture obtained from different areas of the UK (Dr. Brian Moffat, Personal Communications 2006). A waterproof and protective layer of plastic sheeting and gravel was then laid on top. This preparation, by the archaeologists, protected the archaeological layer from extraneous contaminants whilst enabling relatively simple access to this layer for future investigations. Soil samples were obtained by inserting the open end of a sterile 60 ml specimen container (Sterilin, UK) into the excavated archaeological layer creating a 'plug' of soil in the container. This process was carried out by the archaeologist in charge of the excavation (Dr. Brian Moffat, Director of SHARP) so that the appropriate archaeological layer would be exposed and examined. All samples were labelled and sealed in 'zip lock' bags and then stored at 4°C until used.

York latrine samples

Two samples from latrine sediment dating from the Viking era (~AD 800 – 1000) from the city of York, UK (16 – 22 Coppergate, York, UK, Ordinance Survey reference: SE 604 517 GB, Figure 7.3) were provided by the York Archaeological Trust from the 1976 – 1981 excavation of this area (Sample identities 76.81.7 and 99.94.6). The initial sample analysis and formol ether concentration were carried out at the Scottish Parasite Diagnostic Laboratories (SPDL; Stobhill Hospital) by Prof. Huw Smith, with further analysis by Mr. Grant Spence (Senior Biomedical Scientist) then 0.5% trisodium phosphate rehydration / concentration and analysis carried out by myself at the Methicillin Resistant *Staphylococcus aureus* (MRSA) Reference Laboratories, a separate lab on the same hospital site.

Figure 7.2 Excavation of Soutra Aisle.

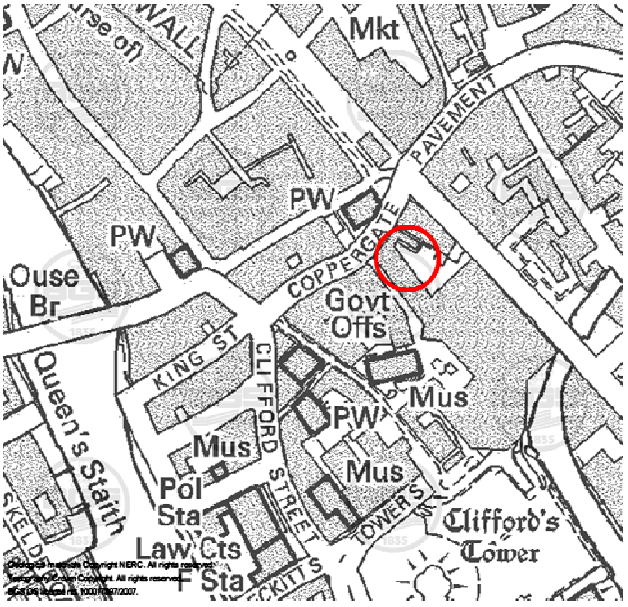


Photograph of the area excavated and marked in yellow (**A**). The diagrammatic representation of the excavation area (**B**). An area of ~1 m square (Yellow square) was marked off and excavated to a depth of 1.5 m. Samples were removed in a horizontal line from a 0.3 m exposed area of the archaeological layer (S1 – S6) and from 0.3 m under the surface of the imported protective 'Top Soil' layer (S7 and S8) by Dr. Brian Moffat in 60 ml sterile sample containers.

Figure 7.3 Location of York.

The location of York within the UK is marked on the map © Crown Copyright 2007 www.ordnancesurvey.co.uk/**getamap** Image produced from Ordnance Survey's Get-a-map service. Image reproduced with permission of Ordnance Survey and Ordnance Survey of Northern Ireland. Scale: 1:625,000.

Figure 7.4 Location of 16 – 22 Coppergate in York.



The area of the Coppergate excavation carried out between 1976 and 1981 is circled in red. This site revealed the buried remains of Viking age York, now recreated in the Jorvik Viking Centre. Image reproduced with permission of Ordnance Survey and Ordnance Survey of Northern Ireland and the British Geological Survey. Scale: 1:1250.

7:3.2 Sample preparations

Formol ether concentration of samples

A further modification of the simplified Ritchie's formol-ether method for the concentration of cysts, ova and larvae in faeces (Ridley and Hawgood 1956) used by Allen and Ridley (1970) was used to concentrate the samples for microscopic examination. This method achieves an increase in the concentration of parasite cysts and ova by 15 to 50 fold over the simplified Ritchie's formol-ether method. Whilst this increase in concentration is dependent upon the parasite sought (i.e. helminth ova) this further modification by Allen and Ridley (1970) provides a good concentrate of protozoan cysts and helminth ova which are diagnostically satisfactory (Allen and Ridley 1970).

A sample of approximately 0.5 – 1.0 g (pea sized) was added using an applicator stick to a 15 ml conical tube containing 7 ml of 10% formalin (10% of a 40% solution of formaldehyde in water). The sample was then thoroughly comminuted with the applicator stick. The resulting suspension was then filtered through a 355 µm aperture size 38 mm diameter sieve [355 µm aperture size, 38 mm diameter is equivalent to 36 mesh British Standard (BS 410-86) or 40 mesh American Standard (ASTM E11-81)] into a beaker and the filtrate poured back into the tube allowing the efficient separation of parasites from larger particles. Debris trapped on the sieve was discarded and both the sieve and the beaker washed thoroughly in running tap water between each sample.

A 3 ml aliquot of diethyl ether (or ethyl acetate) was added to the formolinised solution, the neck of the tube sealed with a rubber bung and shaken vigorously for 30 sec then centrifuged at 750 x ***g*** for 60 sec.

In a normal faecal concentrate there would be a fatty plug present in the sample after centrifugation and this would be discarded along with the fluid both above and below it by inversion, leaving only a few drops behind to resuspend the pellet in. With the historical samples, no fatty plug was observed and the supernatant could be readily decanted.

The resuspended pellet could then be either examined microscopically, or 1 ml of distilled water could be added, the container sealed and the suspension kept for further examination at a later date without risk of drying out.

Trisodium phosphate rehydration and resuspension of samples

The following procedures were carried out in a laboratory that had no prior contact or history of working with protozoan parasites, in a building unattached to the SPDL (MRSA reference laboratory). A sample of approximately 0.5 – 1.0 g (pea sized) was added using an applicator stick to a 50 ml centrifuge tube containing 10 ml of 0.5% trisodium phosphate (prepared from a 5% w/v stock solution in distilled water). Where York samples were investigated, 0.1 g was added to the tubes. The applicator stick was used to 'grind up' the York and Soutra samples to aid softening. Each tube was then sealed and left at 5°C for 8 weeks to soften and vortexed weekly to resuspend the sample. After softening the samples were centrifuged at 1100 x **g** for 15 min. The supernatant was removed and stored in a fresh 50 ml centrifuge tube and the pellet resuspended in 2.5 ml of fresh 0.5% trisodium phosphate.

Immunomagnetic separation (IMS) of samples

A 500 µl volume of resuspended pellet from a volume 2.5 ml of either a formol-ether concentrate or 0.5% trisodium phosphate concentrate was added to 9500 µl of distilled water in Dynal L10 tubes to give a final volume of 10 ml.

For each 10 ml sample, the protocol for the commercially available IMS kit was followed. Briefly, the following quantities of buffers (Dynal IMS kit, Invitrogen Ltd, Paisley UK) were added to the tubes: 1 ml of 10 x SL^{TM}- Buffer A and 1 ml of 10 x SL^{TM}- Buffer B. Anti-*Cryptosporidium* Dynabeads are resuspended by vortexing, then 100 µl of resuspended beads were added to the Dynal L10 tubes, containing the sample concentrate and SL^{TM} buffers. The tubes were then attached to a rotating mixer, rotated at 15 – 25 rpm for 1 h at room temperature (RT).

The tubes were removed from the rotator and placed in a magnetic particle concentrator (Dynal MPC-6), with the flat side of the tubes facing towards the

magnet. The tubes were rocked through approximately 90°, tilting the cap-end and the base-end of the tube up and down in turn per second for 2 min, with the flat side of the tube facing downwards. Oocysts in the sample will be bound to the paramagnetic beads through antigen binding at the oocyst surface, with the oocyst-bead complexes magnetically attracted to the back of the tube, appearing as a black line. In order to retain the oocyst–bead complex, the tubes were carefully decanted to remove the supernatant whilst still in the magnetic particle concentrator, with the supernatant being decanted into suitable containers in case of magnetic failure and the complex being accidentally decanted.

The tubes were then removed from the Dynal MPC-6 and the beads-oocyst complex resuspended in 1ml of 1 x SL^{TM} - Buffer A by adding 900 µl of 1 x SL ^{TM} - Buffer A into the tubes, mixed gently and then transferring all of the liquid from the Dynal L10 tubes to a labelled 1.5 ml microcentrifuge 'snap cap' tube. The remaining material was resuspended by shaking the glass L10 tube three times and by the addition of 100 µl of 1 x SL ^{TM} - Buffer A to the tube and then transferred to the microcentrifuge tube.

The microcentrifuge tubes were placed in a magnetic particle concentrator (Dynal MPC-S), with the magnetic strip placed in the vertical position. The tubes were rocked through approximately 180° for 1 min with one rocking movement per sec. At the end of this step, the bead-oocyst complexes should form a clear 'dot' on the back of the tubes. Supernatants were aspirated from the tubes using a pipette and thin yellow pipette tips (P200 yellow pipette tips [Cat. No. 739290, Greiner Bio-One Laboratories), taking care not to disturb the bead-oocyst complexes attached to the wall of the tubes adjacent to the magnet. After the removal of the supernatant, the oocysts were dissociated from the beads firstly by removing the magnetic strip from the Dynal MPC-S and then 50 µl of 0.1 M hydrochloric acid (HCl) was carefully added to the microcentrifuge tube which was capped then vortexed for 5 – 10 sec. The tubes were returned to the Dynal MPC-S and allowed to stand in a vertical position for 5 min at RT then vortexed for 5 - 10 sec.

Once all the sample had been allowed to settle at the base of the tube the magnetic strip was re-inserted into the Dynal MPC-S and the apparatus allowed to stand undisturbed for a minimum of 10 sec in a tilted position so that the magnetic beads could attach to the Dynal MPC-S magnet.

To a labelled, single welled slide, 5 µl of 1.0 N sodium hydroxide (NaOH) was added to the well and the entire sample from the tube was transferred to the slide, taking care not to disturb the beads at the back wall of the tube. This sample was then mixed with the NaOH on the slide.

The sample was allowed to air dry at RT and the prepared sample slides stored at RT in a dry box, or in a refrigerator at 4 – 8°C until stained with a commercial fluorescein isothiocyante conjugated monoclonal antibody for *Giardia* cysts and *Cryptosporidium* oocysts (G-C FITCmAb; Giardia/Crypto-Cel Immunofluorescence [IF] Test, TCS Biosciences) and DAPI.

Alternatively, the beads-oocyst complexes were not dissociated after the removal of supernatant. A 50 µl volume of lysis buffer was added in the place of HCl and the sample was used for direct DNA extraction.

Fluorescence staining of samples

Sample turbidity was such that it was necessary to dilute the initial sample concentrate for fluorescence staining. A 100 µl aliquot of a trisodium phosphate prepared sample concentrate was added to a 1.5 ml microcentrifuge tube containing 1.4 ml of distilled water to create a 1:15 dilution for each sample. A 50 µl aliquot of this diluted trisodium phosphate prepared sample, or formol ether prepared sample, was added to the well of a labelled single well slide and allowed to air dry. The slide/s were then fixed in methanol for 1 min and allowed to air dry. To each slide, 50 µl of G-C FITCmAb were added to the methanol fixed sample and then incubated at 37°C in a humidity chamber for 60 – 90 min.

Using the methods of Smith *et al*. (2002), a Büchner type aspirator was used to aspirate the excess G-C FITCmAb from the slide by carefully tilting the slide and placing the aspirator tip close to, but not touching, the fluid in a well. To the well, 50 µl of PBS was added and incubated at RT for 1 min before aspirating. This was repeated once more and 50 µl DAPI of a 1:5000 dilution (in PBS) from a working stock of 2 mg ml^{-1} was added and incubated at RT for 2 min. This was then aspirated from the well in the manner previously described for the removal of excess G-C FITC mAb. A single drop of deionised water was added to the well with a plastic pastette and left on the well for 1 min at RT, then aspirated.

Once the slide air dried at RT, 10 µl of mounting medium was applied to the well and a 22 mm x 22 mm coverslip was gently lowered onto the well and allowed to settle before being sealed using clear nail varnish. Two slides for each concentration method per sample were analysed.

DNA extraction from oocyst positive slides or from IMS bead-oocyst complexes

A small volume of liquid nitrogen was transferred, from the designated storage area, in a small laboratory thermos to the DNA extraction laboratory. After microscopic examination, the slides were prepared for DNA extraction using the method of Nichols, Campbell and Smith (2006).

Briefly, the slides were placed on absorbent tissue and the nail varnish sealing the coverslips was removed by swabbing the perimeter of the coverslip with a cotton swab impregnated with nail varnish remover to soften the sealing nail varnish. The softened nail varnish was then scraped away from the coverslip-slide interface onto the absorbent tissue by using the opposite end of the swab. A clean scalpel blade was used to lever a corner of the coverslip from the slide surface and then the coverslip was gently lifted off the slide. The opposite end of the coverslip was gently held to avoid sideways movement of the coverslip or slide. Once lifted, the coverslip was inverted and placed onto the absorbent tissue. The Teflon-coated area of the slide surrounding the well was dried with a small piece of folded absorbent tissue and then

10 µl of lysis buffer (LB; 50 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0, 0.5% sodium dodecyl sulfate) was pipetted onto the well of the slide. The entire surface of the well was scraped with a sterile 1 µl bacteriological inoculation loop (Nunc, UK) and, once scraped, the loop was placed on a support so that it did not rest on a potentially contaminated surface.

Residual LB was recovered by tilting the slide to an angle of ~45° from the horizontal and aspirating the fluid which collected at the bottom of the well by placing the tip of a P20 Gilson pipette fitted with a filter-tipped pipette tip close to the fluid. The scraped sample in LB was pipetted into an appropriately labelled 0.5 ml screw-cap microcentrifuge tube. A further 10 µl volume of fresh LB was deposited onto the sample well using a clean pipette tip and the sample was scraped using the same inoculation loop. As before, the loop was placed on a support so that it did not rest on a potentially contaminated surface. All liquid was removed from the well as previously described and the slide rotated through 180° with the slide-scraping steps repeated, twice again. The final volume of the sample amounted to ~40 µl. The loop was carefully snapped, by pressing it against the inner wall and the rim of the microcentrifuge tube and left inside the tube, which was capped. Swabs, gloves and absorbent tissues were disposed of immediately after each sample was removed from a slide.

IMS bead-oocyst complexes which were not dissociated with HCl were suspended in 50 µl of LB and DNA extraction was performed as described by Nichols & Smith (2004) and Nichols, Campbell and Smith (2003).

Briefly, tubes containing the scraped suspensions and the end of the bacteriological loop or IMS Bead-complexes in 1.5 ml microcentrifuge tube tubes were immersed in liquid nitrogen for 1 min and thawed in a 65°C water bath for 1 min. This freeze-thawing cycle was repeated 15 times and every five cycles, tube contents were mixed by gentle rocking. Each sample was centrifuged at 14,000 x ***g*** for 10 sec to ensure that all the sample lysate was deposited at the base of the tube. Lysates were transferred into a clean tube containing 2 µl of proteinase K (5 mg ml^{-1}) and

incubated at 55°C for 3 h in a water bath. Following incubation, capped tubes were centrifuged at 14,000 x ***g*** for 10 sec to ensure that all the sample lysate was deposited at the base of the tube. Samples were incubated at 90°C for 20 min in a water bath to denature proteinase K, chilled on ice for ~1 min and then centrifuged at 14,000 x ***g*** for 5 min at RT. All supernatant (~30 ml) was transferred to a clean, labelled tube and stored at -20°C until used.

Sample preparation for gel electrophoresis

Either 3 or 15µl of gel loading buffer (bromophenol blue in 1 x Tris-acetate-EDTA buffer (Appendix 7) and 50% glycerol) were added to clean 0.5ml microcentrifuge tubes and 10 or 50µl of PCR product, respectively, were mixed with the gel loading buffer. The sample was loaded in the gel wells with a micropipette immediately before the electrophoresis run. DNA molecular weight markers, (100bp DNA ladder, Invitrogen, UK), was diluted to give a final concentration of 0.5µg per 10 µl in TE buffer (10mM Tris-HCl, 1mM EDTA, pH 8.0) and bromophenol blue loading buffer and stored at 4°C. Ten µl of the prepared DNA ladder dilution were run alongside the samples as DNA size references.

7:3.3 Sample analysis

Microscopic examination of sample concentrates – light microscopy

Initial examination of the sample concentrates occurred under light microscopy conditions (Olympus BH-2 epifluorescence microscope equipped with Nomarski DIC optics) where a 10 – 25 µl aliquot of either a formol ether or trisodium phosphate prepared concentrate was placed on a clear glass slide and a 22 mm x 22 mm cover slip placed on top. This was then sealed with clear nail varnish to prevent the sample from drying out. Scanning of this 'wet' mount commenced in the upper left hand corner of the cover slip under the x20 objective lens, working across the slide from left to right, 1 field width at a time, until the upper right-hand edge of the sample was reached. The microscope stage was then moved so that the field was moved down 1 field height and then continued to scan across the slide from right to left, field by field, until the right-hand edge of the sample was reached. This continued until the slide had been completely scanned for parasite ova. During this

period of observation, the fine focus was adjusted continuously so that the depth of the sample was also scanned and any parasites discovered were identified, counted and photographed (ColorView Soft Imaging System MTV-3 camera and analySIS® software, GmbH, Germany), with any identification being carried out under the x40 objective lens and confirmed by another member of the laboratory.

Microscopic examination of sample concentrates – Fluorescence stained samples

Epifluorescence microscopy using ultra-violet (UV) excitation (excitation 355nm, emission 450 nm) was used to determine the presence of the DAPI stained sporozoite or trophozoite nuclei. A blue filter block (excitation 490 nm; emission 510 nm) was used to visualise G-C FITCmAb emissions. Nomarski DIC optics was used to determine the presence of internal morphology. Under fluorescent conditions, G-C FITCmAb stained *Cryptosporidium* spp. oocysts appear as apple green circular discs 4 – 6 µm in diameter and *Giardia* spp. cysts appearing as apple green ovals ~8 x 12 µm in size. Any object located under the x40 oil immersion objective showing the above fluorescence was further examined using the x100 oil immersion objective. The fluorescent image was related to the bright field image and its size measured with the eye-piece graticule. DAPI intercalates with the nuclei of the sporozoites within viable or non-viable *Cryptosporidium* oocysts and the trophozoites of *Giardia* cysts and fluoresces as a bright sky blue colour. The demonstration of up to four DAPI stained nuclei in an object of a comparable size to an oocyst was counted as a possible oocyst of *Cryptosporidium*. All objects that appeared to correspond to either *Cryptosporidium* oocysts or *Giardia* cysts were assessed for their size, shape and the presence of internal organelles by DIC with the x40 and x100 (oil) objective lenses. All fluorescent stained slides were of the single well type and were scanned in the manner previously described for light microscopy. Two slides for each concentration method per sample were analysed.

Polymerase chain reaction (PCR) of samples

Two 2-step nested 18S rRNA gene PCRs for species identification were used and 2 genetic loci were selected. These are published nested PCR methods using the 18S

rRNA *Cryptosporidium* gene to determine *Cryptosporidium* species and genotypes (Diagnostic = Nichols & Smith 2004; Nichols, Campbell and Smith 2003 & 2006; Xiao = Xiao *et al*. 1999 & 2001).

All PCR megamix reactions were prepared in a laminar flow cabinet presterilised by UV light in an area designated as the pre-PCR lab, DNA extraction was prepared in a separate lab within the same building (designated as the DNA extraction lab) and DNA lysates were added to the PCR reaction volumes in the extraction lab and PCR 1 products were added to the PCR 2 reaction mixtures in a third lab (designated as the PCR lab) to reduce cross contamination. All these procedures were performed in the MRSA reference laboratory.

For the 2 nested 18S PCR reactions total volumes of either 50 µl or 100 µl were used, containing premixed reagents at final concentrations of 200 nM of the forward and reverse primers (MWG BioTech, Milton Keynes, UK), DNAse and RNAse free water (Sigma-Aldrich Company Ltd, Dorset, UK), 200 µM of each of the four deoxynucleoside triphosphates (dNTPs; Invitrogen Ltd, Paisley, UK), bovine serum albumin (BSA) at 4 mg ml^{-1} (Sigma-Aldrich), 20% Tween 20 (BDH), $MgCl_2$ (2.5 mM) and *Taq* polymerase (0.5U) in 1 x PCR buffer IV, (ABgene, Epsom, UK) (Nichols & Smith 2004; Nichols, Campbell & Smith 2003 and 2006; Xiao *et al*. 1999 and 2001) and the PCR megamix dispensed into 0.5 ml thin walled tubes. Both 18S reactions incorporated polyvinylpyrrolidone (PVP) in the PCR megamix to reduce potential PCR inhibition (Nichols, Campbell and Smith 2006). A stock solution of 10 mg ml^{-1} of PVP in 50 mM Tris-HCL (pH 8.0), used at a final concentration of 2 mg ml^{-1} of PVP was incorporated directly into the PCR mixture.

Varying volumes of DNA lysate or first PCR product were used according to the PCR assay being carried out. Both DNA lysate and the first PCR product are used at a volume at which each PCR assay will work optimally (i.e. a maximum of 5 µl of DNA lysate and 3 µl of PCR 1 product). DNA lysate in the first PCR should not exceed one tenth of the total reaction volume, as this ratio has been optimised for the immediate inactivation of SDS, present in the DNA lysate, by Tween 20

(Goldenberger *et al*. 1995; Nichols & Smith 2004). Also possible PCR reaction inhibitors present in the DNA lysates prepared from the Soutra and York samples need to be eliminated. In order to do this, lesser volumes of lysate are used in the reaction and the 'shortfall' in volume made up with DNAse and RNAse free water so that the inhibitors become diluted. For the first amplification (PCR 1) 5 or 3 µl of DNA lysate was added to the reaction mixture to achieve a total volume 50 µl. For the second amplification (PCR 2) 3 µl of PCR 1 product are used with the reaction mixture to achieve a total volume of 100 µl.

Controls were used at each stage of the PCR with *C. parvum* (IOWA isolate) oocyst lysate diluted at one sporozoite per µl, 10^4 oocysts ml^{-1}, used as a positive *Cryptosporidium* DNA control. In the pre-PCR lab a negative control consisting of DNAse and RNAse free water was prepared and added to a PCR reaction. Two other negative controls were prepared consisting of LB and added to reaction volumes in the DNA extraction lab. Set protocols were followed for the preparation and addition of samples to the reaction volumes. The first negative (DNAse and RNAse free water) was prepared in the pre-PCR lab and then the second negative (LB) was prepared followed by all the test samples, the third negative control (LB) and finally the positive control.

All PCR amplifications were performed in a GeneAmp PCR Thermal Cycler (model 9700, Applied Biosystems, Perkin-Elmer, UK). The PCR product was visualized by gel electrophoresis on either 2% or 1.4% agarose gels (Appendix 7) and stained with ethidium bromide on a UV transilluminator (UVT-20 M/V; UV emission at 302 nm; Herolab). Gels were photographed using the Gel Doc 2000 system (Bio-Rad, United Kingdom) equipped with the Quantity-One software for gel documentation and quantitation of PCRs.

Oligonucleotide primers

Oligonucleotides were purchased from MWG BioTech (UK) and reconstituted to 100µM according to manufacturer's instructions. Primers were added to the PCR at

concentration recommended in published methods. The sequences of primers used for each PCR assays are as follows:

18S.a reaction (Diagnostic) the following primers were used:
PCR1 forward primer was WR494F (5'-TGA GTK AAG TAT AAA CCC CTT TAC-3') and its reverse primer being XiaoR1 (5'-CCC ATT TCC TTC GAA ACA GGA-3'). This would produce a PCR product of ~655 bp. In the PCR2 reaction the forward primer used was CPB-DIAG.F (5'-AAG CTC GTA GTT GGA TTT CTG-3') and its reverse CPB-DIAG.R (5'-TAA GGT GCT GAA GGA GTA AGG-3') giving a product of ~435 bp.

18S.b reaction (Xiao) the following primers were used:
PCR1 forward primer was XiaoF1 (5'-TTC TAG AGC TAA TAC ATG CG-3') and its reverse primer being XiaoR1 (5'-CCC ATT TCC TTC GAA ACA GGA-3'). This would produce a PCR product of ~1,325 bp. In the PCR2 reaction the forward primer used was XiaoF2 (5'-GGA AGG GTT GTA TTT ATT AGA TAA AG-3') and its reverse XaioR2 (5'-AAG GAG TAA GGA ACA ACC TCC A-3') giving a product of ~826 bp.

For each pair of primers, the respective thermocycler programmes used are shown in Appendix 8.

Enzymatic digestion of positive secondary PCR products and RFLP analysis

The positive secondary PCR products of the nested-18S.a assay were digested with specific restriction enzymes and the fragments separated by electrophoresis in 2% agarose gels.

The 18S.a positive PCR2 amplicon were initially digested, simultaneously, with the restriction enzymes *Ase*I (New England Biolabs, UK) and *Dra*I (Invitrogen Ltd). These restriction enzymes allow the profile of *Cryptosporidium* species to be determined and are shown in Appendix 8; Table 1.

The buffers used with each enzyme were provided by the suppliers and used according to their instructions. Restriction digests were performed in 50 µl total reaction volume of which 20 µl consisted of the positive PCR product and 2 µl (20U) of each restriction enzyme. The tubes were incubated at 37°C for 2 h and the digested products separated in 2% agarose gels stained with ethidium bromide.

Gel electrophoresis

The electrophoresis apparatus consisted of submerging gel tanks, a selection of combs (Anachem Ltd, UK) and a power supply (Electrophoresis power Supply 500/400, Pharmacia, UK). The gel was first submerged in 1 x TAE buffer, then samples were loaded into the wells and run at 80V for 1 h (1.4% gels) or up to 3 h (2% gels). The 3 h gel runs were required to obtain good separation of small DNA fragments.

7:4 RESULTS

Microscopic examination of sample concentrates – light microscopy

Sample concentrates from both methods (formal-ether and trisodium phosphate) were examined microscopically for helminth ova and protozoan cysts and oocysts. The presence of *Trichuris* spp. ova were found for both York samples with up to 6 ova being found in each slide for York sample 99.94.6. However, in York sample 76.81.7 only one *Trichuris* spp. ovum was found in all the analyses. A single *Trichuris* ovum was found in the Soutra samples (S4). The same York sample that contained *Trichuris* ova (99.94.6), also showed the presence of a single infertile *Ascaris* ovum after analysing 2 slides. These findings were consistent between both sample preparation methods of formol-ether concentration and 0.5% trisodium phosphate rehydration.

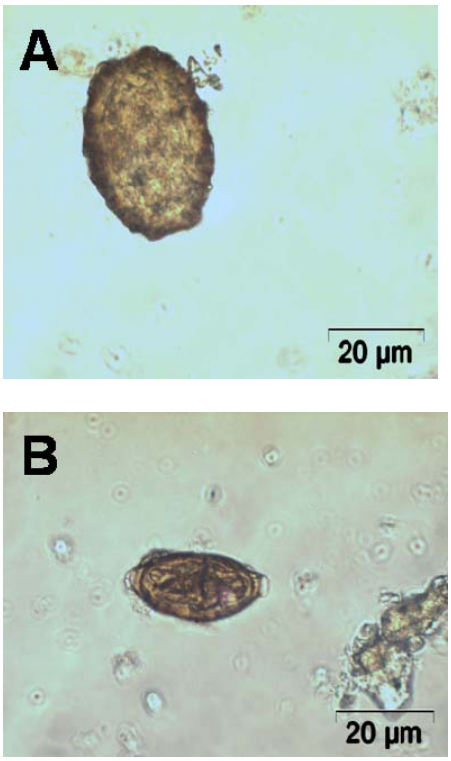


Figure 7.5 Parasites discovered in Soutra and York.
Brightfield microscopy of an infertile *Ascaris* ovum (**A**) and a *Trichuris* ovum (**B**). Parasites are viewed under a total magnification of x250 for *Ascaris* and x500 for *Trichuris*. Representative photographs of each parasite from 2 replicate slide analysis for both formol-ether concentration and 0.5% trisodium phosphate rehydration methods are shown. The parasites were viewed under brightfield conditions with an Olympus BH-2 microscope fitted with Nomarski DIC optics. Photographs were taken (copyright© SPDL) using a ColorView Soft Imaging System MTV-3 camera and analySIS® software.

Microscopic examination of sample concentrates – Fluorescence stained samples

G-C FITCmAb was used to visualise *Cryptosporidium* oocysts and *Giardia* cysts in both formol-ether concentrates and 0.5% trisodium phosphate concentrates. Initially, formol-ether concentrates were examined and oocysts of *Cryptosporidium* identified by FITC staining and the inclusion of the nuclear fluorogenic dye, DAPI. Identification was confirmed by another member of SPDL staff (Mr G. Spence). Sample 76.81.7 from York was found to contain 4 oocysts on the slides showing the characteristic size (5 µm x 5 µm) and shape (spherical) of oocysts which also stained bright apple green with G-C FITCmAb. Present in the oocyst were 4 DAPI stained nuclei indicated by 4 sky blue 'dots' (Figure 7.6 C – F). A third method of microscopic identification is usually undertaken by viewing viewed under Nomarski DIC microscopy (Olympus BH2 microscope, x40 and x100 objectives) however, DIC views were unobtainable due to debris obscuring the sample. Soutra samples were also found to contain oocysts of *Cryptosporidium*.

From 2 Soutra samples prepared by 0.5% trisodium phosphate rehydration/concentration (S2 and S3), a total of 3 G-C FITCmAb stained, roughly spherical bodies of the correct size for *Cryptosporidium* were found (Figure 7.7). They could not be positively identified as *Cryptosporidium* oocysts when viewed under UV-DAPI filters, as these brightly FITC stained objects did not show the presence of sporozoite nuclei. This was partly due to the high 'background' staining of DAPI most likely due to the presence of bacteria in the sample and partly due to debris obscuring the field of view (Figure 7.7).

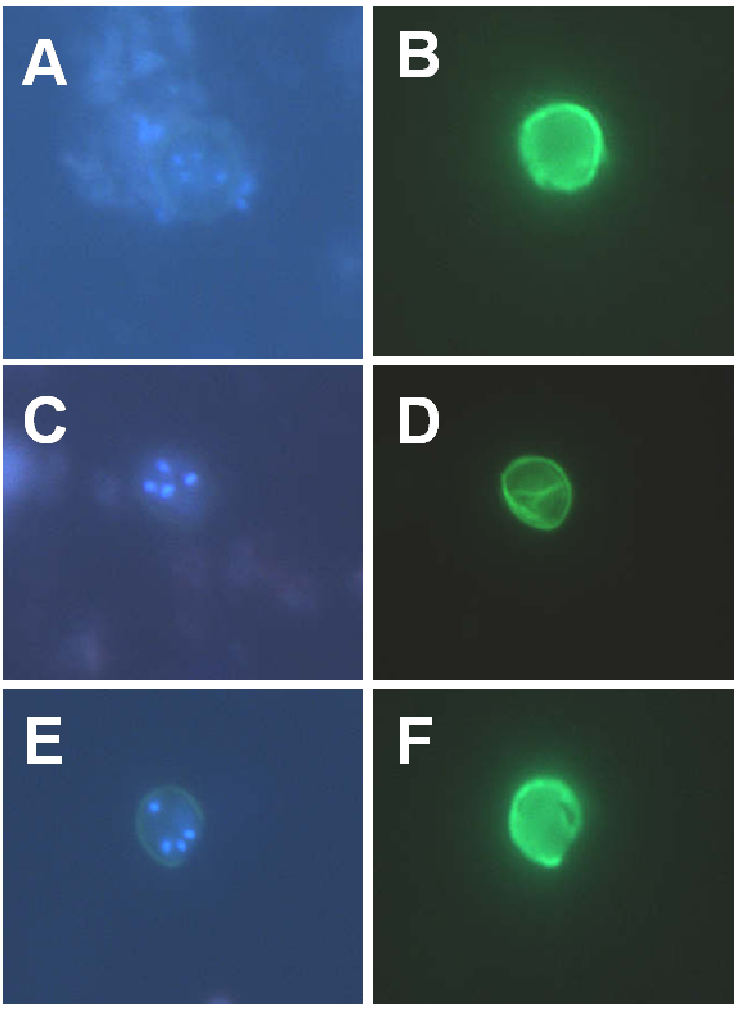


Figure 7.6 Fluorescence staining of formol-ether sample concentrates from York and Soutra.
G-C FITCmAb staining of *Cryptosporidium spp*. oocysts (B, D & F) and DAPI (A, C & E) showing 4 distinct nuclei characteristic of *Cryptosporidium* oocysts. Soutra (A & B) and York (C – F). The parasites were viewed under epifluorescence microscopy using UV excitation (excitation 355nm, emission 450 nm) with an Olympus BH-2 microscope fitted with Nomarski DIC optics at a total magnification of x1250. Pictures were taken (copyright© SPDL) using a ColorView Soft Imaging System MTV-3 camera and analySIS® software.

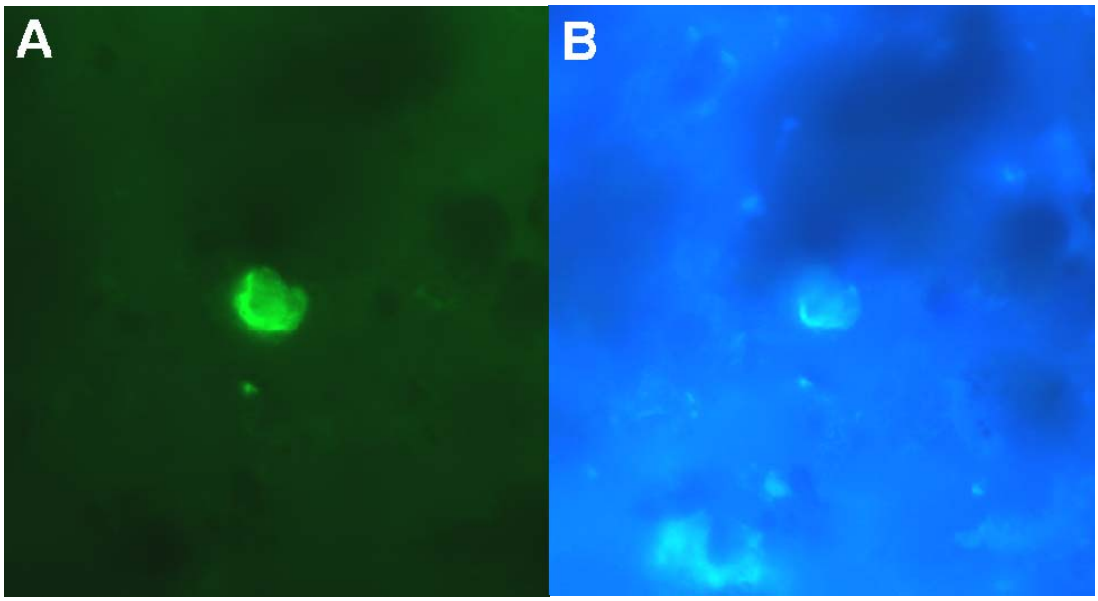


Figure 7.7 Fluorescence staining of 0.5% trisodium phosphate sample concentrates from Soutra.
G-C FITCmAb staining of *Cryptosporidium spp*. oocysts **A**) and DAPI **B**) showing staining of an oocyst like body, having the correct size for *Cryptosporidium spp*. oocysts (5 µm x 5 µm). DAPI staining of sporozoites was not visible and confirmation that the object was a *Cryptosporidium spp*. oocyst could not be made. The oocyst like body is shown under epifluorescence microscopy using UV excitation (excitation 355nm, emission 450 nm) with an Olympus BH-2 microscope fitted with Nomarski DIC optics at a total magnification of x1250. Pictures were taken (copyright© SPDL) using a ColorView Soft Imaging System MTV-3 camera and analySIS® software.

PCR analysis of samples

As intact *Cryptosporidium* oocysts containing sporozoites, as visualised by DAPI incorporation, were found in the formol-ether preparations, DNA lysates were prepared from the positives slides. Both 18S.a and 18S.b methods were employed to identify *Cryptosporidium* DNA and if possible, differentiate the species. Analysis of the G-C FITCmAb and DAPI positive slides from formol-ether concentrates showed non-specific amplication of PCR products (Figure 7.8). By using slurries from 0.5% trisodium phosphate prepared concentrates it was hoped that this non-specific amplification or inhibitory / interference effect would be avoided. Preparation of these slurries, DNA lysate, as well as the PCR reactions were all carried out in a laboratory which had never handled *Cryptosporidium* DNA or oocysts.

These slurries were subjected to IMS and lysates were prepared directly from oocyst-bead complexes. They were then analysed for the presence of *Cryptosporidium* DNA using the 18S.a method of Nichols, Campbell and Smith (2003) with either 5 µl or 3 µl of lysate. This latter concentration of lysate was used to minimise the concentration of any inhibitory products from the sample. All the Soutra samples were very weakly positive for *Cryptosporidium* DNA (Figure 7.9) and further analysis to confirm this was carried out. Since the positive sample amplicons were of the same size, a second gel using selected DNA which had been prepared from 5 µl lysates was run on 2% agarose to obtain better separation of small DNA fragments. This would help to confirm that the Soutra sample amplicons which were weakly positive for *Cryptosporidium* spp. DNA were of an equivalent size to that of the *C. parvum* DNA positive control. Soutra samples S4 and S8 were chosen for this gel as well as both York samples together with a control negative and a control positive. Both the Soutra samples remained very weakly positive and were found to be ~450 bp in size. This is of comparable size with the *C. parvum* DNA positive control, suggesting that the samples are positive for *Cryptosporidium* DNA (Figure 7.10). However, further analysis would be required to confirm this and so enzymatic digestions of the samples were performed.

Enzymatic digestion of positive secondary PCR products and RFLP analysis

The positive secondary PCR products of the nested-18S.a assay were digested with specific restriction enzymes and the fragments separated by electrophoresis in 2% agarose gels. Enzymatic cleavage was used to confirm that the investigated samples were positive for *Cryptosporidium* DNA and also to determine which species they might be.

RFLP analysis of the amplicons, by simultaneous digestion with the enzymes *Ase*I and *Dra*I, demonstrated amplicons of a size compatible with *Cryptosporidium* species. However, the fragmented PCR product amplicon lengths are slightly different from *C. parvum* (as shown with the control DNA sample) and further analysis would be required to make a further identification of the species, possibly using sequencing technologies (Figure 7.11).

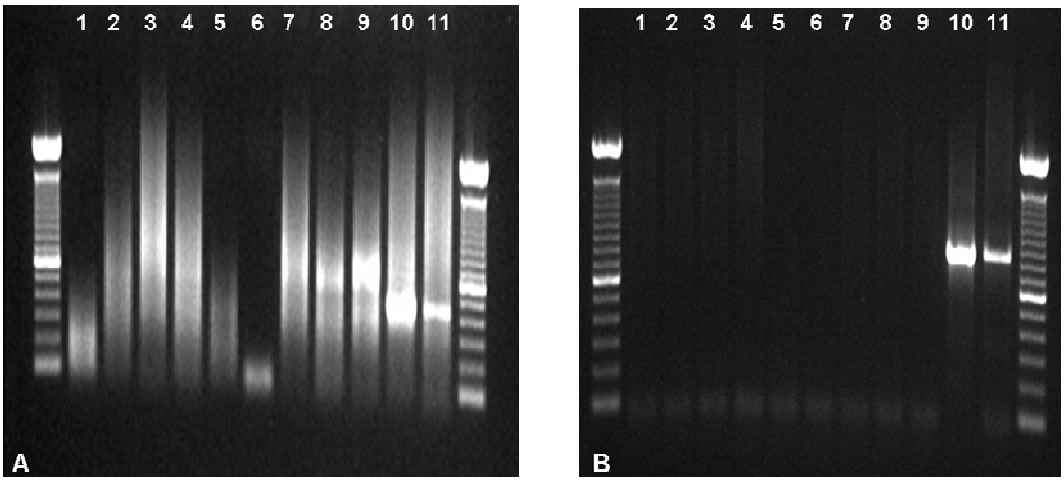


Figure 7.8 PCR analysis of *Cryptosporidium* oocyst positive slides.
Five oocyst positive samples from Soutra (panels A and B, lanes 3 – 7) and 2 from York (panels A and B, lanes 8 – 9) were analysed for the presence of *Cryptosporidium* DNA using **A**) 18S.a method of Nichols, Campbell and Smith (2003) and **B**) 18S.b method of Xiao *et al*. (1999, 2000a). *Cryptosporidium* DNA negative (panels A and B, lanes 1 & 2) and *Cryptosporidium* DNA positive controls (panels A and B, lanes 10 & 11) were also used. Unfortunately, parasite DNA was not amplified in this experiment. Samples were run on a 1.4% agarose gel at 80V for 1 h stained with ethidium bromide.

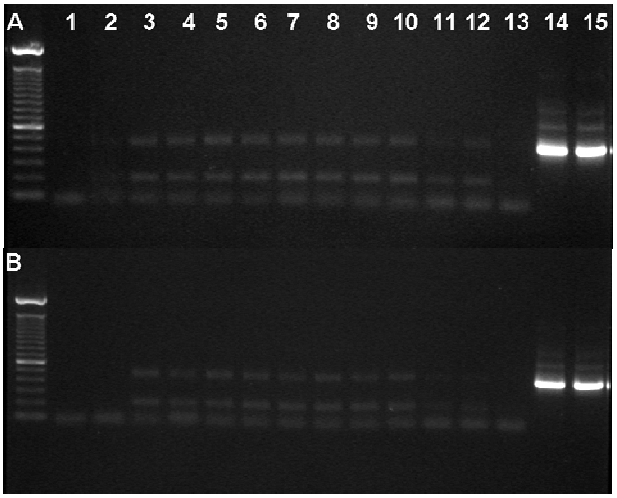


Figure 7.9 PCR analysis for *Cryptosporidium* DNA in 0.5% trisodium phosphate rehydrated/concentrated samples.
DNA lysates were prepared from all the sample concentrates from 0.5% trisodium phosphate rehydration/concentration. They were then analysed for the presence of *Cryptosporidium* DNA using the 18S.a method of Nichols, Campbell and Smith (2003) with either **A**) 5 µl of lysate or **B**) 3 µl of lysate. *Cryptosporidium* DNA negative (lanes 1, 2 and 13) and *Cryptosporidium* DNA positive controls (lanes 14 & 15) were used. Lanes 3 – 10 represent all the samples obtained from Soutra (S1 – S8) and lanes 11 and 12 represent both York samples (99.94.6 and 76.81.7, respectively). All the Soutra samples were very weakly positive for parasite DNA and further analysis to confirm this was then carried out. Samples were run on a 1.4% agarose gel at 80V for 1 h stained with ethidium bromide.

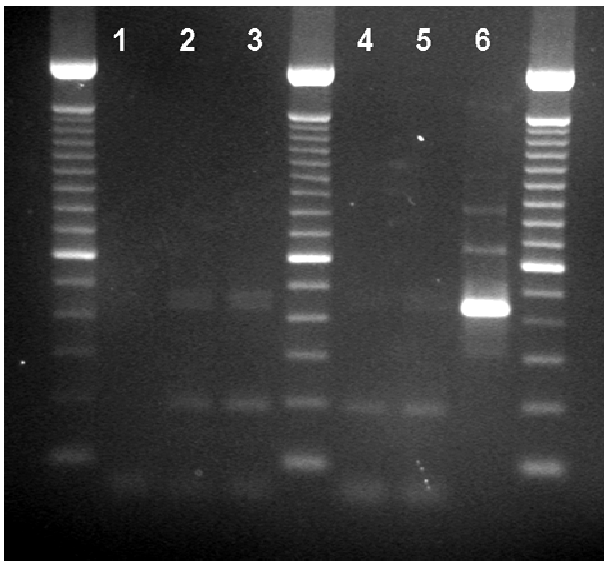


Figure 7.10 PCR analysis for *Cryptosporidium* DNA from selected 0.5% trisodium phosphate rehydrated/concentrated samples.
Samples which were thought to be positive for *Cryptosporidium* DNA using the 18S.a method of Nichols, Campbell and Smith (2003) were run on a second gel to enhance separation of small DNA fragments and confirm their size compared with a known *C. parvum* DNA positive control (lane 6). Two samples from Soutra (S4 and S8; lanes 2 and 3 respectively) and both York samples (99.94.6 and 76.81.7; lanes 4 and 5, respectively) were prepared from all the sample concentrates from 0.5% trisodium phosphate rehydration/concentration. *Cryptosporidium* DNA negative (lane 1) was also used. All the Soutra samples were very weakly positive for parasite DNA and were of a size which compared to the positive control. Samples were run on a 2% agarose gel at 80V for 2 h stained with ethidium bromide.

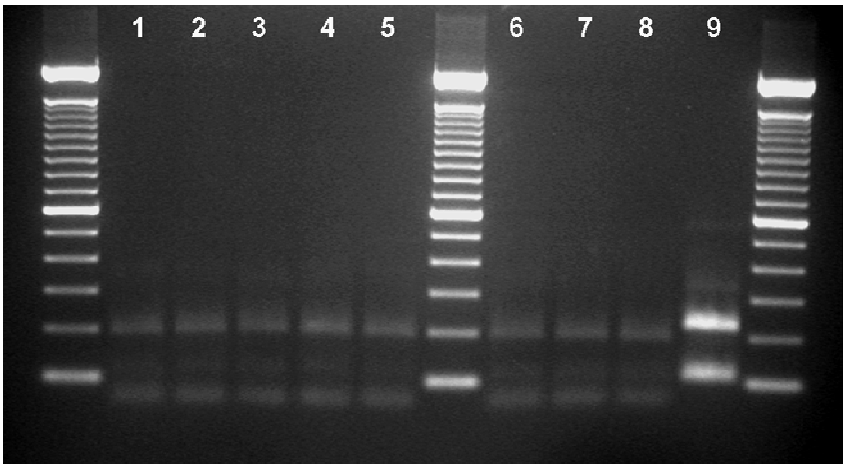


Figure 7.11 A 2% agarose gel electrophoresis analysis of amplicons after simultaneous digestion with restriction enzymes *Dra*I and *Ase*I.
Samples which were thought to be positive for *Cryptosporidium* DNA using the 18S.a method of Nichols, Campbell and Smith (2003) were digested with the enzymes *AseI* and *DraI*. All 8 samples from Soutra (lanes 1 – 8) were digested along with a *C. parvum* positive DNA control (lane 9). Samples were run on a 2% agarose gel at 80V for 2 h stained with ethidium bromide.

7:5 DISCUSSION

Palaeoarchaeological analyses are performed to provide some clues as to the dietary behaviours, nutrients, health conditions and the natures of occupation to supplement other archaeological evidence of historical habitation. Desiccated, or mineralized, coprolites and soils, recovered from archaeological sites, are routinely analysed for parasites. Parasites and/or ova have been found in archaeological materials, usually from the periods associated with humans (Araujo & Ferreira 2000).

The positive identification of parasites in humans from faeces is a routine operation in many laboratories around the world. The methods by which they occur are generally through the use of microscopy (fluorescence and bright field) and sometimes by the use of molecular techniques. However, samples from antiquity provide different and often complicated challenges for the scientist, where sample type can often be calcified and dehydrated and requiring weeks to months of rehydration before the sample can be examined. In clinical samples one can have an idea of what particular parasites to look for, as the sample will often be clearly defined and is accompanied with clinical notes telling the patient history. In ancient samples, the patient is long dead, leaving behind, at times, only scanty evidence of existence. The challenge for the scientist is to be able to not just identify organisms but simply to find them in samples.

In this study the latrine sediments of a Viking settlement in York (UK) and soil samples from a 14th century AD hospital cess pit were examined microscopically and molecularly for the presence of intragastric parasites, with a special emphasis for on the protozoan parasites *Cryptosporidium* and *Giardia*. Both sample types were found to contain ova from *Trichuris* and one sample from York also contained an ovum from *Ascaris*. All samples were positive for *Cryptosporidium* spp. oocysts by fluorescent microscopy methods. PCR-RFLP analysis of *Cryptosporidium* positive slides indicate that *Cryptosporidium* spp. was found in the Soutra hospital drain.

In western countries, it has been reported that *Ascaris* sp. and *Trichuris* sp. are the most commonly encountered ova of parasites from historical sources (Taylor 1955;

Pike 1967 & 1968; Jones 1982; Aspock, Auer and Picher 1996). They have been reported from as early as the Neolithic, up to the Roman and Saxon eras, in England, Germany, Australia, Holland, Poland, Denmark and Israel. Previous findings from York and Soutra also discovered these ova (Dr. Brian Moffat, Personal Communications 2006; Anon. 1999; Kenward & Hall 1995) and the results provided in this thesis are able to confirm these previous findings.

The possible presence of intact oocysts in both the York and Soutra samples prepared from formol-ether concentration was at first an exciting prospect, as the only other report of *Cryptosporidium* oocysts being found in antiquity was from the coprolites isolated from Peruvian mummies (Ortega & Bonavia 2003). The identification of *Cryptosporidium* spp. oocysts was possible using G-C FITCmAb directed against known epitopes. This suggests that not only are the glycoprotein epitopes found on the oocyst wall extremely robust, being able to survive at least 4,300 years BP (Ortega and Bonavia 2003), but that they are also highly conserved through this period of time, with little or no change to their structure.

DNA analysis of historical samples is now, in the past 10 years, being used to determine the diagnosis and confirmation of infectious diseases (Araújo *et al*. 1998). An example of this has been the successful diagnosis of trypanosomiasis in South American mummies. Initially, the infection was proposed in mummies through the microscopic examination of amastigote pseudocysts in mummified tissues (Rothhammer 1985; Rothhammer *et al*. 1985; Fornaciari *et al*. 1992). The diagnosis of this disease in South American mummies was then successfully confirmed using PCR techniques (Guhl *et al*. 1997 & 1999; Ferreira *et al*. 2000).

In order to determine the presence and identification of *Cryptosporidium* spp. DNA in the archaeological samples, two 2-step nested 18S rRNA gene PCRs (Diagnostic and Xiao) for species identification were used. These are published nested PCR methods using the 18S rRNA *Cryptosporidium* gene to determine *Cryptosporidium* species and genotypes (Diagnostic = Nichols & Smith 2004; Nichols, Campbell and Smith 2003 & 2006; Xiao = Xiao *et al*. 1999 & 2001). Unfortunately no

Cryptosporidium DNA was amplified using this method from the formol-ether concentrates. It was thought that perhaps the presence of formol-ether in the samples was at a concentration sufficient to inhibit the PCR reaction. All these examinations were originally prepared and analysed at the SPDL.

Whilst the above slides were prepared in the SPDL using standard bench aseptic techniques, this facility does handle various types of *Cryptosporidium* samples (in water and faeces) on a regular basis and so the equipment used and the prepared formol-ether concentrates could not be absolutely guaranteed to be free of *Cryptosporidium* oocysts. In order to ensure that the parasites observed were not some contamination of the sample by oocysts in the atmosphere or on the equipment, a secondary lab was used to freshly prepare concentrates (MRSA reference laboratory). This facility was in the same hospital grounds as the SPDL, had no history of working with *Cryptosporidium* and was in another building almost half a mile from the SPDL. Also, due to the inhibition of the PCR reaction by formol-ether concentration, samples were prepared in a solution of 0.5% trisodium phosphate to rehydrate followed by centrifugation of the samples to concentrate them. It is not known whether this solution would have any adverse affect on the PCR reaction, but it has been routinely used in the rehydration of samples for the identification of parasites microscopically since the 1940's (Van Cleave & Ross 1947; Pike 1968).

Where the presence of oocysts was suspected under fluorescence microscopy in Soutra samples reconstituted in 0.5% trisodium phosphate and examined, the nested 2 step 18S rRNA assay (a highly sensitive assay being able to amplify and identify as few as 5 oocysts on a single slide) and RFLP analysis of the samples was carried out to confirm or deny the presence of *Cryptosporidium* DNA. Using the diagnostic primers (PCR 1 = WR494F [forward] + XiaoR1 [reverse]; PCR2 = CPB-DIAG [forward and reverse]) and method of Nichols and Smith (2004) and Nichols, Campbell and Smith (2003), amplicons of a length comparable to *Cryptospordium* DNA was demonstrated, suggesting its presence, albeit very weakly in all the samples from Soutra, but not York. Further analysis to identify the species of *Cryptosporidium* provided by the CPB-DIAG primers was performed by RFLP using

the digestion enzymes *Dra*I and *Ase*I. In this analysis, the presence of *Cryptosporidium* DNA was further confirmed but the species / genotype could not be determined. These amplicons elicited the same size of digest fragments, indicating that the species of *Cryptosporidium* found was of the same type in all the Soutra samples. These initial results would indicate the first reporting of *Cryptosporidium* in archaeological findings in Europe.

The identification of the *Cryptosporidium* species found in the Soutra samples could have implications as to the health of individuals from 14th C AD at that site, as of the 19 valid species (Chapter 1; Table 1.4), 8 are known to infect humans and of those 8, C. *parvum* and *C. hominis* predominate infections (Smith 2008; Smith *et al*. 2007; Feltus *et al*. 2006; Leoni *et al*. 2006; Nichols, Campbell and Smith 2006; Caccio *et al*. 2005; Ryan *et al*. 2004; Xiao *et al*. 2004). The identification of *Cryptosporidium* species in the Soutra samples could provide hints as to the source of *Cryptosporidium*, i.e. *C. meleagridis* is known to infect turkeys (Slavin 1955), whereas *C. baileyi* infects chickens (Current, Upton and Haynes 1986) and *C. parvum* infects man and other mammals (Ng *et al*. 2008; Zintl *et al*. 2008; Feltus *et al*. 2006; Xiao *et al*. 2004; reviewed by O'Donoghue 1995; Current *et al*. 1983; Tzipori *et al*. 1981; Nime *et al*. 1976).

The correct identification of the origin of the specimens can pose special problems in the validity of data. When faecal material is not obtained from a mummified body, it can be difficult to ascertain its origin. Parasites found in faecal material or even in the intestines of preserved human remains have to be approached with a degree of caution when interpretating the recorded parasitism. For example, the parasite ova or cysts may be present in the human digestive tract through the consumption of the parasites' definitive host. The ingestion of contaminated meat or offal was thought to be the reason behind the finding of *Eimeria mira*, a protozoan parasite of the Red Squirrel, in the intestines of the Bog Man of Grauballe dated between 1540 and 1740 years BP (Hill 1990).

In this study the *Cryptospordium* amplicons observed in the Soutra samples were found to be of similar size and would indicate a common source. This is potentially a worry for the correct identification of this parasite at the site. Two of the stated positive samples were taken from topsoil used to infill the site at the time of the original excavation and was sourced from another area in Britain (Dr. Brian Moffat, Personal Communications 2006). There is the possibility that the parasites, or their DNA, was present in the imported soil and has 'leached' with the action of water downwards to the archaeological layer. This would account for the presence of uniform fragment lengths in the PCR-RFLP analysis. However, further PCR-RFLP analysis is required to positively identify the species of *Cryptosporidium* found. With this technology and the with the aid of sequencing the DNA found, it may be possible to prove or disprove this hypothesis, that there are *Cryptosporidium* oocysts / DNA present in the samples and that they are of a different species from those found in the imported top soil. The use of larger sample volumes in the original softening and concentration stage may help increase the quantity of oocysts to be found and subsequently, the quantity of DNA to be amplified allowing the sequencing of the DNA amplicons. If these techniques determine that the *Cryptosporidium* DNA present in the imported topsoil samples is different from the archaeological samples, this will be the first reported finding of *Cryptosporidium* in a European archaeological site.

CONCLUSIONS

This series of experiments were designed to determine if PEOs and PRFEs had any effect on the viability of 3 protozoan parasites, covering 2 Phyla and 3 Families. The organisms tested were the enteric pathogens *C. parvum*, *G. duodenalis* and the intracellular protozoan *T. cruzi*. These parasites are responsible for debilitating diseases in millions of people worldwide (an estimated 280 million for *Giardia* and 16 – 18 million for *T. cruzi* and over 500, 000 humans thought to have been infected with *Cryptosporidium* during 1983 – 2003 in waterborne outbreaks). Whilst chemotherapeutic methods of treatment may be available, there are various factors involved in drug management (such as increasing parasite resistance, side effects of drugs and their cost) that require new sources of therapeutic compounds to be made available.

With *Cryptosporidium*, only one drug, nitazoxanide, has been used to any great effect and is currently licenced for use with immunocompetent patients ≥ 1 years of age in the USA (Rossignol 2006; Smith & Corcoran 2004). All other attempts at chemotherapy have had mixed results both *in vitro* and *in vivo*, but none of over 200 drugs tested were effective or specific enough in its action for chemotherapeutic use. Whilst this parasite causes self limiting diarrhoea in immunocompetent hosts, potentially fatal consequences can occur when it infects the very young, the old or immunocompromised.

T. cruzi does currently have effective chemotherapy, however, the 2 drugs of choice, benznidazole and nifurtimox, are either expensive, difficult to obtain (Weir 2006), have poor patient compliance due to lengthy treatments, have side effects and parasite resistance to the drug (Buckner *et al*. 1998). Also, the greatest difficulty in treatment is that both drugs are effective in treating the acute phase of infection, which can be difficult to diagnose, but not the chronic. It is during the chronic phase of infection that most complications and fatalities occur. The most frequently used treatment is the management of the clinical manifestations of the disease, such as

pacemakers being fitted for heart arrhythmias, chronic heart failure, transplantation and surgery for megacolon.

Of the 3 parasites, *Giardia* is the least complicated to treat by chemotherapy. In the majority of cases, a short treatment regimen of tinidazole (Fasigyn®) (2 g once in adults) or metronidazole (Flagyl®) (250 mg three times daily 7 days for adults) are 80% to 95% effective, respectively (Petri 2005). However, it is the common side effects of these drugs which are problematic. They have been variously described as nausea, headaches, diarrhoea, abdominal cramps, a strong metallic taste, dizziness, dark discoloured urine and a disulphiram-like reaction when taken with alcohol (nausea and vomiting). These problems can be such that patients will often endure the parasite, rather than attempt to cope with the side effects of chemotherapy.

The aims of the project were: i) to determine if any of the tested PEOs and PRFEs had antigiardial, antitrypanosomal, or anticryptosporidial effects *in vitro*; ii) to determine the minimum concentration of PEOs and PRFEs required to inhibit the growth of *G. duodenalis* trophozoites and *T. cruzi* epimastigotes and to affect the excystation of *C. parvum* oocysts; iii) to determine the mode of action of the PEOs and PRFEs and compare the inhibitory properties of the PEOs and PRFEs with metronidazole; iv) to determine potential active constituents from the composition of effective PEOs and their mechanism of action through microscopic and biochemical means so that their potential as novel drugs could be evaluated; v) to determine the validity of the historical / traditional use of bilberries for the treatment diarrhoea and vi) to determine the presence or absence of protozoan parasites in a medieval hospital drain and a Viking latrine.

It was demonstrated that PEOs influence the morphology of *G. duodenalis* trophozoites and *T. cruzi* epimastigotes over time. Also, PEOs can induce the spontaneous excystation of *C. parvum* oocysts *in vitro* in the absence of recognised *in vitro* excystation triggers, with the exception of time and elevated temperature. Bright field microscopy was used to identify morphological changes in the parasites. PEOs incubated at a final concentration of 0.02% for 24 h caused the swelling,

distortion, blebbing and increased killing of trophozoites *in vitro* and the contraction, rounding and flagellar internalization of epimastigotes (Chapter 2; Figure 2.3 and Chapter 4; Figure 4.5 and Table 4.2). All PEOs were equally effective at 0.02% in reducing parasite viability by 100%. In the *Cryptosporidium* experiments, the concentration required to induce spontaneous excystation in the presence of PEOs was 0.2%, 10 times greater than that for *Giardia* trophozoites and *Trypanosoma* epimastigotes. This is possibly due to *Cryptosporidium's* robust oocyst wall which is known to be resistant to environmental pressures (Nichols, Paton and Smith 2004; Robertson, Campbell and Smith 1992) and resistant to disinfection (Angus *et al*. 1982; Blewett 1989; Campbell *et al*. 1982; Korich *et al*. 1990; Li *et al*. 2004; Pavlasek 1984; Peeters *et al*. 1989).

The changes to the morphology of both *Giardia* and *Trypanosoma* parasites indicate an action of PEOs by interaction with the cell / plasma membrane. The formation of giant membrane protrusions may or may not be a part of some kind of programmed cell death, but may also be due to changes to the structure of the cell / plasma membrane caused by the PEOs or their constituents. Changes in the osmoregulation of the cell could also be indicated by the swelling and rounding up of *Giardia* trophozoites (Chapter 2; Figure 2.3 B, D and E), which has also been observed by Pérez-Arriaga *et al*. (2006) with curcumin treated trophozoites and by Campanati & Monteiro-Leal (2002) with metronidazole treated *Giardia* trophozoites. Interaction of PEO with the cell / plasma membrane is quite possible due to the lipophilic properties of PEOs and their ready diffusion across cell / plasma membranes (Boyom *et al*. 2003). Large intracytoplasmic vacuole formation in myrtle treated trophozoites (Chapter 2; Figure 2.3 D) indicate that some kind of programmed cell death may have been initiated as this has also been observed in curcumin treated trophozoites (Pérez-Arriaga *et al*. 2006).

The inhibition of *T. cruzi* epimastigote growth by ajoene from garlic has been shown to cause changes in the phospholipid composition of the parasites cell membrane, leading to the previously most abundant phospholipids, phosphatidylcholine (PC), to become the least abundant, with its immediate precursor (phosphatidylethanolamine)

becoming the most abundant. This suggests that ajoene exerts its effects by inhibiting the final stage of PC biosynthesis and thus, alter the phospholipid composition of the cell. This could also be observed ultrastructurally with a concentration dependent alteration in intracellular membranous structures such as mitochondrial swelling and the aggregation or fusing of membrane vesicles (Urbina *et al*. 1993). Studies such as this may help further elucidate the morphological action of PEOs tested in this thesis.

Protein analyses of myrtle (*Myrtus communis* L.) and elemi (*Canarium luzonicum* L.) oils demonstrate that they can inhibit the expression of a 37 kDa protein and the upregulation of other proteins in *Giardia* trophozoites. Whilst these particular proteins remain unidentified, it clearly shows that these oils can not only affect the morphology of the cell but also its biology.

Nuclear enlargement in PEO treated *Giardia* trophozoites was also observed, indicating a possible intracytoplasmic action similar to that found in metronidazole treatment (Chapter 2; Figure 2.3 A). Also demonstrated was blebbing and cytoplasmic granulation, suggestive of apoptosis, an intriguing proposition as the cornerstone for apoptosis, the mitochondrion, is absent in this protist (Chose *et al*. 2003).

Unlike the vegetative forms of parasites, oocysts of *C. parvum* were more resistant to the effects of the majority of the PEOs tested. However, sweet fennel (*Foeniculum vulgare* var. *dulce* BATT. & TRAB.), geranium (*Pelargonium graveolens*) and palmarosa (*Cymbopogon martinii* (ROXB.) J.F. WATSON) were found to have the greatest effects in inducing spontaneous excystation, with palmarosa being determined to have the greatest activity. This PEO was able to induce excystation of oocysts at levels comparable to that of the maximised *in vitro* excystation protocol (Chapter 3; Figure 3.1).

The antitrypanosomal and antigiardial actions of PEOs were found to be concentration dependent. When incubated in different concentrations of PEOs, 2 were found to have the lowest MICs and were effective against both *Giardia*

trophozoites and *Trypanosoma* epimastigotes. These oils were myrtle and elemi. Increasing the concentration of *Giardia* trophozoites also required a slight increase in the concentration required for 100% killing. This may have connotations for the treatment of giardiasis where up to 1 $x10^8$ cysts can be excreted per gram of faeces (Roxström-Lindquist *et al*. 2006) indicating a high burden of *Giardia* trophozoites present in the small intestine. The maximum concentration of trophozoites used in this thesis was 5 x 10^7 ml^{-1} which is likely to be lower than that found in an active infection. This would then indicate a greater concentration of oil would be required to exert the same antigiardial activity *in vivo* than with the *in vitro* concentrations.

Titration of the PEOs identified the MICs required for the complete killing of parasites. Studies demonstrated that the oils myrtle and elemi had the greatest antigiardial and antitrypanosomal effects with MICs of 0.005% (50 μg ml^{-1}) in *G. duodenalis* trophozoites and at least 0.00125% (12.5 μg ml^{-1}) in *T. cruzi* epimastigotes, with the added effect of killing > 97% at lower concentrations of 0.000313% (3.13 μg ml^{-1}) for myrtle and 0.000625% (6.25 μg ml^{-1}) for elemi with *T. cruzi* epimastigotes. For *Giardia* trophozoites, these oils did have greater activity than metronidazole when examining the MIC data (myrtle and elemi MIC = 0.005% [50 μg ml^{-1}]; metronidazole MIC = 67 μg ml^{-1}) but not when the 90% and 50% inhibitory concentrations were investigated. The activity with *T. cruzi* epimastigotes, however, revealed these oils exhibited an IC_{50} in the range given for benznidazole (myrtle IC_{50} = 0.0002% [2 μg ml^{-1}]; elemi IC_{50} = 0.0003% [3 μg ml^{-1}]; benznidazole IC_{50} 0.73 – 4.4 μg ml^{-1}) identifying these oils as a possible source of novel chemotherapy for American trypanosomiasis. This low concentration can also have potential implications for therapeutic use, where myrtle oil has been shown to cause no detectable skin sensitization when diluted, with both myrtle and elemi capable of being used on the skin with low risks of allergic reactions (Tisserand & Balacs 1995). This would indicate that it is possible to use these PEOs safely to kill epimastigotes on the skin surrounding the bite wound of the Reduviid bug, thus preventing the entry of viable epimastigotes into the body.

Studies by Mikus *et al*. (2000) and Hoet *et al*. (2006) have demonstrated that the individual constituents of PEOs may exert trypanocidal actions. The terpenic constituents' terpinen-4-ol, E-nerolidol and linalool were all effective at inhibiting *T. brucei* growth *in vitro* and were highly selective for the parasite and not mammalian cells. The constituent terpinen-4-ol was also found to be as selective as the drug Suramin® (Mikus *et al*. 2000).

In those experiments, the terpenic compounds were found to have a greater affect than that of the whole oil. All the active PEOs in this thesis contain terpenes, but unlike the above examples, they were found to have actions comparable to and sometimes less than that of the whole oil. As synthesis of these compounds are possible and with them having actions in most cases comparable to the whole oil, it may be that the compounds could be manufactured for therapeutic use instead of the large scale production of PEOs. An example of such large scale production with low yield is the production of myrtle oil. In the PEO industry, myrtle oil production can show yields of 0.25 – 0.55% or, in other words, every 100 g of plant material distilled will yield only 0.25 – 0.55 g of PEO. This would obviously require a great deal of plant material to produce large quantities of this oil. This is precisely the problem encountered with production of the antimalarial drug, artemisinin. There is an estimated 150 million treatments available from the current world wide stocks of *Artemisia annua*. However, there is an estimated 300 – 500 million cases of malaria and the time taken to increase the production from plant material to drug is approximately 12 – 18 months (WHO 2007). Also, the yield of the active constituent is naturally low in the plant (< 1%) which has lead to the formation of a project to increase the yield of artemisinin from the plant to reduce the costs of production (the Centre for Novel Agricultural Products *Artemisia* research project, Dept. of Biology, University of York, UK).

The speed at which the PEOs tested demonstrated effects on *T. cruzi* epimastigotes, could have some bearing on the potential application of any of the PEOs as novel antitrypanosomal drugs. A rapid antitrypanosomal effect may be useful in limiting the risk of infection. With American trypanosomiasis, the bite of an infected

Reduviid bug is a means of entry for *T. cruzi* epimastigotes / metacyclic trypomastigotes. Access is via the bite wound, with epimastigotes / metacyclic trypomastigotes present in the urine and faeces of the insect contaminating the wound when the host scratches the irritated area. All the PEOs investigated in this project showed antitrypanosomal activity after 2 h of incubation *in vitro*. As the epimastigotes were killed within 2 h, a topical application of PEO directly to the bite and its surrounding area may help clear the skin of potentially infective *T. cruzi* epimastigotes / metacyclic trypomastigotes, thus reducing the risk of infection.

A secondary benefit of some PEOs is that they may actually reduce the irritation accompanied with the bite, helping to alleviate the pruritus which will reduce the compulsion to scratch the affected area. Evidence to support this theory is the action of TTO to reduce histamine induced inflammation and oedema in humans and mice (Brand *et al*. 2002; Koh *et al*. 2002). As histamine is released to cause the swelling, redness and itchiness associated with insect bites it is thought that the ability of the oil and its major constituent terpinen-4-ol, to reduce histamine induced inflammation can be of benefit in controlling the discomfort of insect bites. Also, PEOs can be applied topically, being used safely in aromatherapy at concentrations between 1 and 30% in inert carrier oils such as almond oil (Tisserand & Balacs 1995).

Considering that the current chemotherapy of Chagas' disease is effective only during the acute phase of infection which can be difficult to detect, the prevention of entry to viable epimastigotes / metacyclic trypomastigotes may help in the overall treatment and management of *T. cruzi* infection. Alternatively, plant oils may also be used as insect repellents. By reducing contact with infected Reduviid bugs, one can limit the chances of infection. Myrtle oil, along with many other PEOs, contains citronellol which is in use today in many 'natural' insect repellents.

Myrtle, elemi, α-pinene and limonene could all be used internally for giardiasis providing they can be delivered to the intestine without being metabolised en route, or being adsorbed elsewhere. A drug which can be fast acting and with a greater retention time within the target area (in the case of *G. duodenalis*, the small intestine)

would be of great benefit in the reduction of the quantity of drug taken with increased efficacy. Data on the bioavailability of plant oils or their constituents are, however, scarce.

An alternative to the use of PEOs are the phenolic compounds found in berries. Polyphenol-rich extracts of berries were found to reduce *G. duodenalis* trophozoite viability. Of particular interest were 4 extracts from the same Family (Rosaceae). These were cloudberry (*Rubus chamaemorus*), artic bramble (*Rubus arcticus*), strawberry (*Fragaria vesca*) and blackberry (*Rubus fruticosus*). All 4 at concentrations of 167 μg ml^{-1} killed 100% of trophozoites within 24 h (Chapter 6; Figure 6.1). However, cloudberry showed the greatest antigiardial action being able to kill 96 ± 4.4% of trophozoites at 67 μg ml^{-1}, a concentration comparable to that of metronidazole (Chapter 6; Figure 6.2). Whilst cloudberry is known to contain high levels of ellagitannins, experiments with its degradation product, ellagic acid, demonstrated no antigiardial activity (Chapter 6; Figure 6.3).

As the PRFEs investigated are devoid of organic acids and the ellagic acid was found not to affect trophozoite viability, the potential remaining classes of phenolic compounds in PRFEs with antigiardial properties could be the flavonoids, lignans, stilbenes or condensed tannins (Puupponen-Pimiä *et al*. 2005a).

One such class of constituent, the flavonoids, have over 4000 different flavonoids described, categorized into flavonols, flavones, catechins, flavanones, anthocyanidins and isoflavonoids. Blueberries may be potentially useful for treating giardiasis. Anthocyanins are the major polyphenol constituent of blueberries and appear to be prime candidates for the anti-giardial effects reported in this thesis. Although anthocyanins have low serum bioavailability in humans, they have considerable longevity in the gastrointestinal tract and are known to reach the colon largely undegraded (up to 85% of the initial dose) either following their consumption (Kahle *et al*. 2006) or following *in vitro* simulation of human digestion (McDougall *et al*. 2005). This makes them ideal candidates for a potential drug with intragastric action and also perhaps as a drug for the treatment of cryptosporidiosis. A possible use for

anticryptosporidial activity may be when the sporozoites have invaded enterocytes and during the replicative cycle where the release of the actively motile merozoites from type I schizonts can infect neighbouring cells. By interfering with either their motility or viability the infection could be limited. Investigations into the anti-sporozoite or merozoite capabilities of berry phenols or plant oils could be carried out *in vitro*. For antigiardial effects to occur, only a small quantity of blueberries were required (40 g) and with the antigiardial effect of cloudberry being found to be comparable to that of metronidazole, they could be used as food supplements in the case of giardiasis for its treatment rather than the current drug therapy with its side effects.

However, the anthocyanins are just one of the multitude of different compounds found within plants and for the PEOs tested in this thesis, information on the bioavailability of terpene compounds (the active class of compounds found in palmarosa, geranium, myrtle and elemi oils) is scanty. Further work would need to be carried out in this field before one could properly determine their suitability as novel antigiardial drugs. Nevertheless, the rapid action of the PEOs in this study has shown a potential for novel drug design which could reduce the duration of treatment.

Potentially, there may be applications for sweet fennel oil or *trans*-anethole as a means of excysting *C. parvum* oocysts prior to entry in the small intestine with their 57% spontaneous excystation rate over 4 h. It is well known that sporozoites of *Cryptosporidium* are unable to survive extremes of pH and that excystation of *C. parvum* oocysts in the stomach, which is too acidic for sporozoite survival (optimal pH 6.2, Woodmansee *et al.* 1987) causing sporozoite lysis, thus reducing enterocyte invasion and the subsequent propagation of infection. By utilising the speed at which *trans*-anethole and sweet fennel oil are able to cause spontaneous excystation it may be possible induce this action at a site which is unfavourable to the sporozoite survival, e.g. the stomach.

Further, the slower acting geranium, palmarosa, citronellol and geraniol may also have chemotherapeutic uses for cryptosporidiosis. By inducing the release of sporozoites prior to the release of oocysts in faeces we can limit the propagation of infection, not just in humans as an immediate treatment but also prophylactically in cattle and other livestock, a major source of infection for humans (Caccio *et al*. 2005). Providing that the pharmacokinetics of these compounds are found to be favourable for a sustained exposure in the gut they become prime novel drug candidates. With oocyst secretion lasting up to 4 weeks in humans (Baxby, Hart & Blundell 1985), this slow action (up to 24 h for peak activity) may help limit the propagation of infection through the induction of excystation just prior to release or by weakening the oocyst wall so that it becomes environmentally sensitive.

With the demonstrated action of palmarosa, geranium and their terpenic constituents, geraniol and citronellol (a breakdown product of geraniol in bacterial metabolism), there are possible uses in livestock. By limiting the amount of infected oocysts being passed in faeces it is possible to reduce the risk of potential human infection caused by the faecal contamination of potable water. Using these compounds in animal feed may be one means of control but the treatment of farmyard faecal slurry may also be of benefit especially as geraniol is known to safely and rapidly biodegrade in the soil.

Some hazard information is currently available for the compounds citronellol, geraniol and *trans*-anethole as well as geranium, palmarosa and sweet fennel PEOs. All 3 constituents and PEOs are currently used in food as flavourings. Indeed, the US FDA considers them all as GRAS for food use (FDA Food additive status list 2006; FFDCA 2004). The WHO have assessed that citronellol most likely metabolises to harmless substances based on data on a corresponding substance (geraniol) and that the majority of the compound is eliminated in the urine. Both citronellol and geraniol are used as synthetic flavourings being GRAS (FDA Food additive status list 2006; FFDCA 2004). This may allow their potential use internally as chemotherapeutic compounds.

Historically, many PEOs and extracts, such as TTO, myrrh and clove, have been used as topical antiseptics, or have been reported to have antimicrobial properties (Hoffman 1987; Lawless 1995). Also the use of fruit as medicine in the treatment of diarrhoea has been shown historically (Dr. Brian Moffat, Personal Communications 2006; Webber and Watson 1998; Anon 1825). It is important to investigate scientifically those plants which have been used in traditional medicines as potential sources of novel antimicrobial compounds (Mitscher *et al*. 1987). Samples of soil from a medieval hospital drain demonstrated the possible presence of *Cryptosporidium* spp. oocysts, or at least their DNA (Chapter 7). If their presence could be confirmed and if further analysis of the samples could find plant material associated with this protozoan, it may be possible to identify remedies in use at that hospital for the treatment of cryptosporidiosis, or its symptoms. That is, of course, id the *Cryptosporidium* species can be identified and is 1 of the 8 species which are known to infect humans.

The historical use of blueberries for the treatment of diarrhoea was demonstrated with the juice and polyphenol rich extract of this fruit being able to inhibit *Giardia* trophozoite viability at concentrations to be found in the equivalent of 40 g of blueberries. Also demonstrated were the exceptional properties of one family of fruits (Rosaceae) which had strong antigiardial activity, with cloudberry PRFE in particular being comparable to metronidazole.

This is the first time that such an extensive screening of PEOs and PRFEs has been carried out on *G. duodenalis* trophozoites, *T. cruzi* epimastigotes and *C. parvum* oocysts. This study has shown that PEOs were effective in inhibiting *G. duodenalis* trophozoite and *T. cruzi* epimastigote viability over a 24 h incubation period at final concentrations in the microtitre well of 0.02%. Furthermore, the trophozoites of *Giardia* and epimastigotes of *Trypanosoma* were found to be more sensitive to oils of myrtle and elemi, with a MIC for these PEOs of 0.005% (50 μg ml^{-1}) for *Giardia* and 0.00125% (12.5 μg ml^{-1}) for *Trypanosoma*. Antigiardial actions of PEOs were found to be rapid, with all but myrrh acting within 60 min of incubation. Further analysis of the PEOs suggests that the monoterpenes α-pinene, from myrtle oil and

limonene, from elemi, were the active constituents found within the oil responsible for the killing of *G. duodenalis* trophozoites and *Trypanosoma* epimastigotes *in vitro*. The effect of spontaneous excystation of *Cryptosporidium* oocysts in the absence of all recognised triggers except temperature caused by palmarosa, geranium, sweet fennel oil and the terpenes geraniol and citronellol, would argue that they might be acting in 1 of 2 ways; i) that the PEOs augment the effect of temperature on oocyst excystation by increasing oocyst wall permeability through pore formation or integration with oocyst wall or ii) the reverse hypothesis, that an increase in temperature causes an activation of spontaneous excystation initiated by the PEOs.

All these data indicate that there is potential use for plant oils, plant extracts and their constituents as novel drugs for the treatment of protozoan parasitic infections. Their uses could vary from the intragastric treatment of infection to the topical application of oils / constituents to prevent parasite entry into the blood stream and tissues. Apart from direct treatment of human infection, limiting the propagation of parasites in livestock may also be possible.

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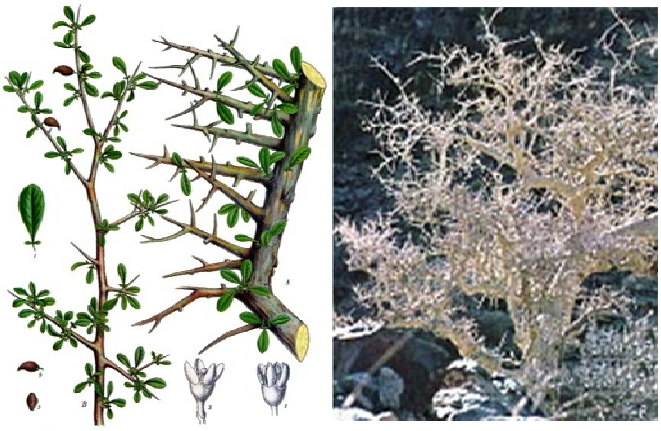
APPENDICES

Appendix 1: Plant essential oils used.

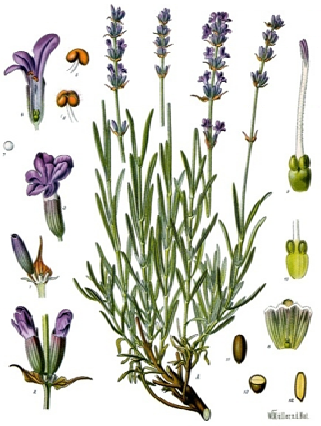
Family: Apiaceae, *Foeniculum vulgare* var. *dulce* BATT. & TRAB. (**Sweet Fennel**). Fennel is an erect growing perennial herb native to southern Europe and the Mediterranean area. Reaching a height of 1.5 metres (5 feet), the plant has yellow flowers on a compound umbel. Approximately 60 percent of the essential oil is located in the fruit/seeds, with the rest in the rays of the umber and other green plant parts. Illustration: Köhler's Medicinal Plants, 1887. Photograph: Wikipedia, Carsten Niehaus, 2005.

Family: Burseraceae, *Canarium luzonicum* L. (**Elemi**). It is a tropical tree from the Philippines that can grow up to 30 metres (98 feet) high. The tree exudes a pale yellow resin when the tree sprouts leaves. The aromatic oil is steam distilled from this resin. Illustration: Köhler's Medicinal Plants, 1887.



Family: Burseraceae, *Commiphora myrrha* (NEES) ENGL. (**Myrrh**). The myrrh plant is a pubescent perennial, up to 90 cm (3 feet) in height, with thin, soft, pinnate leaves which grow in the Middle East and eastern Africa. The dark yellow or reddish brown resin is exuded naturally or from incisions made in the bark with the aromatic oil being distilled from this exudate. Illustration: Köhler's Medicinal Plants, 1887. Photograph: Anon.

Family: Geraniaceae, *Pelargonium graveolens* (**Geranium/Rose Geranium**). The woody, perennial herb is native to South Africa and is produced in Egypt, France, the People's Republic of China, Algeria, South Africa, Morocco and Spain. Reaching a height of 1 metre (~ 3 feet), the plant has pubescent, fragrant, green, deeply lobed leaves and rose-colored flowers. The essential oil accumulates in small glands found in the foliage and flowers. Illustration: *Flore des serres et des jardins de l'Europe* by Charles Lemaire *et al*. Gent, Louis van Houtte, 1850, volume 6, plate 607. Photograph: Wikipedia, Eric Hunt, 2005.




Family: Lamiaceae, *Lavandula angustifolia* MILLER (**Lavender**). Lavender is the name for any of several aromatic shrubs, including English lavender, *Lavandula angustifolia* Mill. subsp. *angustifolia* and French lavender, *Lavandula dentata* L. English lavender is native to southern Europe and the Mediterranean area. Cultivated extensively for perfume and ornamental purposes in several European countries, the plant reaches a height of about 1 metre (~ 3 feet), has linear, lanceolate leaves covered with a velvety pubescence and develops blue or purple flowers. Lavender oil is obtained by the immediate steam distillation or solvent extraction of flowers harvested at full bloom. Illustration: Köhler's Medicinal Plants, 1887. Photograph: J-P Anthony, Botanic Gardens, Glasgow, 2004 (copyright© SPDL and QMU).

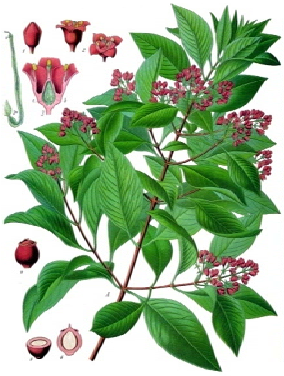
Family: Lamiaceae, *Origanum majorana* L. (**Marjoram**). Marjoram is a tender perennial herb native to North Africa and southwest Asia and naturalized in southern Europe. The plant reaches a height of 50 cm (1.5 feet) and has small, grey-green, ovate leaves, pink or purple flowers and erect, glabrous to tomentose stems. Harvesting is generally accomplished at full bloom and can be done 2 or three times per year with the oil obtained by the steam distillation of plant material. Photograph: Wikipedia, Mark Pellegrini, 2005.

Family: Lamiaceae, *Pogostemon cablin* (BLANCO) Benth. (**Patchouli**).The patchouli plant is a bushy herb reaching 75 – 90 cm (2 – 3 feet) in height with soft, opposite, egg-shaped leaves and square stems. It is a tropical member of the mint family, native to southeast Asia and is cultivated in the East and West Indies. Leaves are harvested several times a year, dried and exported for distillation of the oil, although the highest quality oil is usually produced from fresh leaves, distilled close to the plantation. Photograph: J-P Anthony, Botanic Gardens, Glasgow, 2004 (copyright© SPDL and QMU).

Family: Lamiaceae, *Thymus vulgaris* L. (**Thyme**). Thyme is the general name for the many herbs of the *Thymus* species, all of which are small perennial plants native to Europe and Asia. Common or garden thyme, *Thymus vulgaris* L., is considered the principal type and is utilized commercially for flowering and ornamental purposes. This low-growing woody shrub has grey-green leaves and white, pink, or purple flowers. Harvesting is generally accomplished at full bloom with the oil obtained by the steam distillation of plant material. Illustration: Köhler's Medicinal Plants, 1887. Photograph: J-P Anthony, Botanic Gardens, Glasgow, 2004 (copyright© SPDL and QMU).

Family: Myrtaceae, *Myrtus communis* L. (**Myrtle**). The Myrtle is an evergreen shrub or small tree, growing to 5 metres (15 feet) tall, native to southern Europe and north Africa. The leaves are entire, 3-5 cm long, with a pleasantly fragrant essential oil. The star-like flowers have five petals and sepals and an amazingly large number of stamens. Petals are usually white, with globose blue-black berries containing several seeds. Steam distillation of the leaves and twigs produce the essential oil. Illustration: Prof. Dr. Otto Wilhelm Thomé *Flora von Deutschland, Österreich und der Schweiz* 1885, Gera, Germany. Photograph: Wikipedia, Iorsh, Mount Carmel, Israel, 2004.

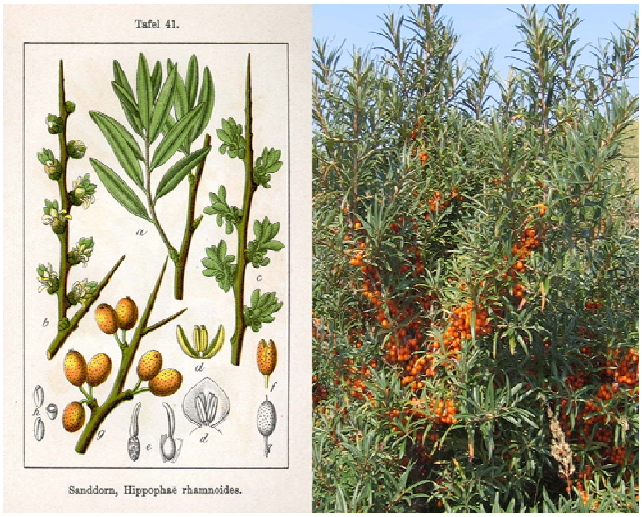
Family: Poaceae, *Cymbopogon martinii* (ROXB.) J.F. WATSON (**Palmarosa**). It is a wild growing, herbaceous perennial green and straw-coloured grass, native to India and Pakistan, with long slender stems, terminal flowering tops and fragrant grassy leaves reaching 1.5 metres (3 feet) in height. It is harvested before the flowers appear and the highest yield is obtained when the grass is fully dried.




Family: Rutaceae, *Amyris balsamifera* L. (**Amyris** or **Balsam Torchwood**). A native of the Caribbean and Gulf of Mexico this perennial small bushy tree with oval, opposite leaves and star-like, pink – red flowers with 4 petals. The essential oil is produced from the steam distillation of the wood. Illustration: Köhler's Medicinal Plants, 1887. Photograph: Anon.


Family: Rutaceae, *Agathosma (prev. Barosma) betulina* (P.J. BERGIUS) BART. & WENDL. (**Buchu**). A low, white or pink-flowered shrubs native to South Africa chiefly found in the south-west region of Cape Colony. The leaves are of a pale green colour, 1/2 to 3/4 inch long, 1/2 inch or less wide, leathery and glossy, with a blunt, strongly-curved tip and finely-toothed margin, with round oil glands scattered through the leaf. The leaves have a strongly aromatic taste and a peppermint-like odour and are steam distilled for their oil. Illustration: Köhler's Medicinal Plants, 1887. Photograph: Anon.

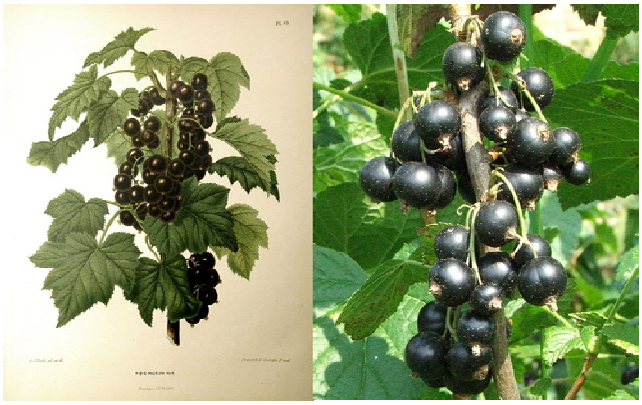
Appendix 2: Soft fruits used.



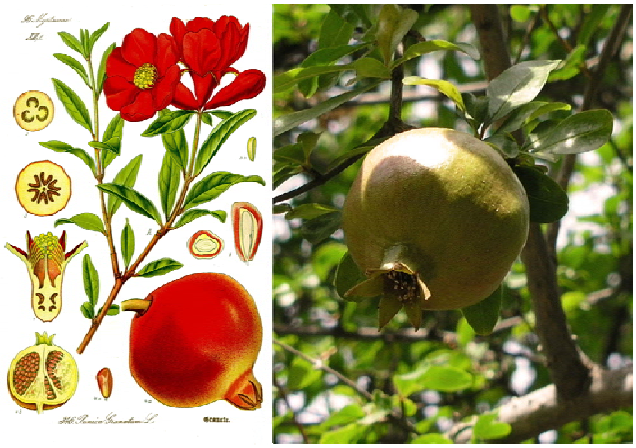
Family: Elaegnaceae, *Hippophae rhamnoides* (**Sea Buckthorn**). There are three species, native over a wide area across Europe and Asia. They reach 0.5 – 6 m tall, rarely up to 18 m in central Asia and typically occur in dry, sandy areas. The *Hippophae rhamnoides* is by far the most widespread, with a range extending from the Atlantic coasts of Europe right across to northwestern China. Sea-buckthorn branches are dense and stiff and very thorny. The leaves are a distinct pale silvery-green, lanceolate, 3 – 8 cm long and less than 7 mm broad. It is dioecious, with separate male and female plants. The male produces brownish flowers which produce wind-distributed pollen. The female plants produce orange berries 6 – 9 mm in diameter, soft and juicy and rich in vitamin C, some varieties are also rich in vitamin A, vitamin E and oils. Illustration: Johann Georg Sturm, *Deutschlands Flora in Abbildungen.* 1796. (Painter: Jacob Sturm). Photograph: Wikipedia, Sander van der Molen, 2006.

Family: Ericaceae, *Vaccinium myrtillus* (**Bilberry**). Bilberry is a name given to several species of low-growing shrubs in the genus *Vaccinium* (family Ericaceae) that bear tasty fruits. The species most often referred to is *Vaccinium myrtillus* L., also known as blaeberry, whortleberry, whinberry (or winberry), myrtle blueberry, fraughan and probably other names regionally. Bilberries are found in damp, acidic soils throughout the temperate and subarctic regions of the world. Illustration: Prof. Dr. Otto Wilhelm Thomé *Flora von Deutschland, Österreich und der Schweiz* 1885, Gera, Germany. Photograph: Wikipedia, Marek Silarski, 2005.

Family: Ericaceae, *Vaccinium vitis-idaea* L. (**Lignonberry/Cowberry**). This is a small evergreen shrub in the flowering plant family Ericaceae that bears edible fruit. It is seldom cultivated, but the fruits are commonly collected in the wild. The native habitat is the circumboreal forests of northern Eurasia and North America, extending from temperate into subarctic climates. Cowberry shrubs of both varieties are typically 10 – 40 cm in height and have a compact habit. The species resembles the related and similar cranberry (*Vaccinium oxycoccus, V. microcarpum* and *V. macrocarpon*), differing mainly in having white (not pink) flowers, with the petals partially enclosing the stamens and stigma (the petals are reflexed backwards in cranberries) and rounder, less pear-shaped berries. Other related plants in the genus *Vaccinium* include blueberries, bilberries and huckleberries. Illustration: C. A. M. Lindman, Bilder ur Nordens Flora, Projekt Runeberg: published 1917–1926. Photograph: Wikipedia, Jonas Bergsten 2005.

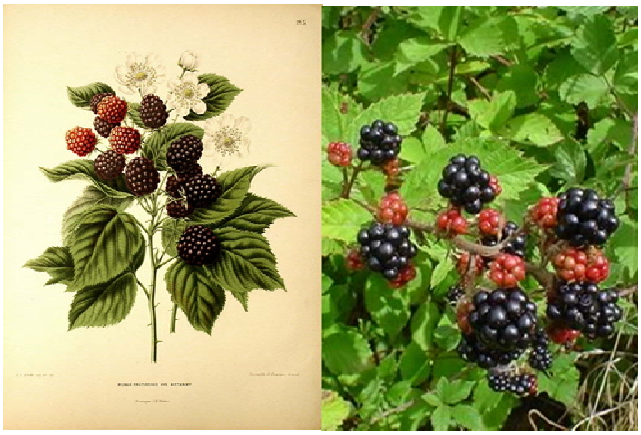


Family: Grossulariaceae, *Ribes nigrum* (**Blackcurrant**). A native of central and northern Europe and northern Asia, it is a small shrub growing 1 – 2 metres (3 – 6 feet) in height. The leaves are alternate, simple, 4 – 9 cm long and broad and palmately lobed with five lobes, with a serrated margin. The flowers are 4 – 6 mm diameter, with five reddish-green to brownish petals; they are produced in racemes 5 – 10 cm long. The fruit is an edible berry 1 cm diameter, very dark purple in colour, almost black, with a glossy skin and a persistent calyx at the apex and containing several seeds. Illustration: Groningen, J.B. Wolters, *Nederlandsche flora en pomona* beschreven en uitgegeven door het bestuur der Pomologische Vereeniging te Boskoop, by K.J.W. Ottolander, A. Koster & C. de Vos (editors) 1876-1879. Chromolithograph by A.J. Wendel. Photograph: Wikipedia, Jerzy Opiola, 2006.



Family: Punicaceae, *Punica granatum* L., (**Pomegranate**). This is a fruit-bearing deciduous shrub or small tree growing to 5 – 8 metres (16 – 27 feet) tall. The pomegranate is native from Iran to the Himalayas in northern India and has been cultivated and naturalized over the whole Mediterranean region including Armenia since ancient times. It is widely cultivated throughout Iran, India, the drier parts of southeast Asia, Malaya, the East Indies and tropical Africa. The leaves are opposite or sub-opposite, glossy, narrow oblong, entire, 3 – 7 cm long and 2 cm broad. The flowers are bright red, 3 cm in diameter, with five petals (often more on cultivated plants). The fruit is between an orange and a grapefruit in size, 7 – 12 cm in diameter with a rounded hexagonal shape and has thick reddish skin and around 600 seeds. The edible parts are the seeds and the red seed pulp surrounding them; indeed, the fruit of the pomegranate is a berry. Illustration: Prof. Dr. Otto Wilhelm Thomé *Flora von Deutschland, Österreich und der Schweiz* 1885, Gera, Germany. Photograph: Wikipedia, Luis Fernandez Garcia, 2003.

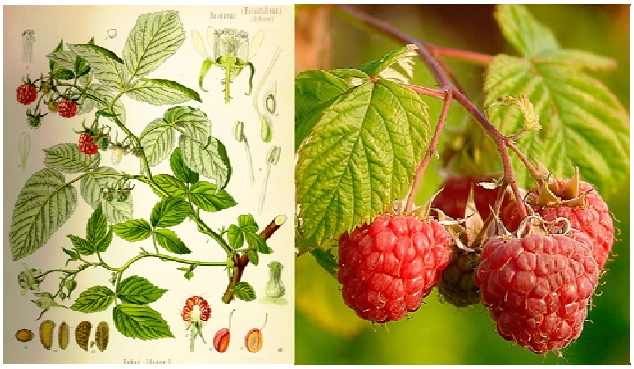
Family: Rosaceae, *Rubus arcticus* (**Artic Bramble**). The arctic bramble can grow up to 30 cms (12 inches) tall. It is spineless and the leaves have three fingers. The plant flourishes in June – July with rose-red flowers which are alone, 2 by 2 or three by three. The flowers are either male or female, with both sexes eventually present in the same plant. The fruits are dark brown/red, very aromatic and maturation begins in August. It is distrubuted in the northern hemisphere mostly in Scandinavian countries, Canada and the more temperate regions of Asia.. Illustration: C. A. M. Lindman, Bilder ur Nordens Flora, Projekt Runeberg: published 1917–1926. Photograph: Kore Wild Fruit Nursery, 2005.



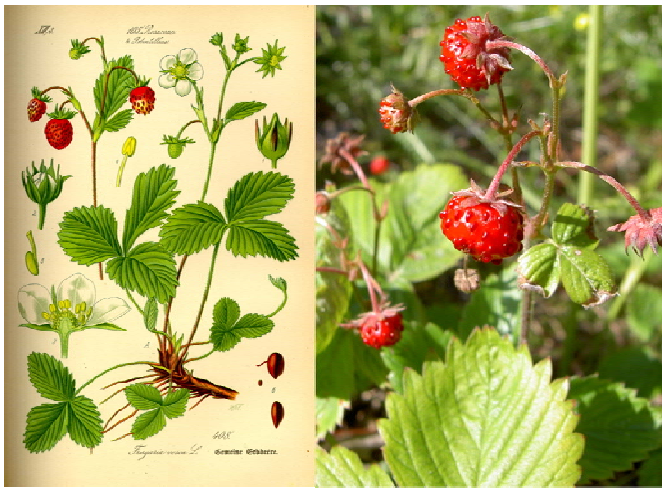
Family: Rosaceae, *Rubus fruticosus* (**Blackberry**). Growing to 3 metres (10 feet) and producing a soft-bodied fruit, this plant is common throughout the temperate regions including North America and Europe. It has palmate or pinnate compound leaves of three to five leaflets with flowers of white or pink appearing from May to August, ripening to a black or dark purple fruit, the blackberry or "bramble". In proper botanical language, it is not a berry at all, but instead an aggregate fruit of numerous drupelets. Illustration: *Nederlandsche flora en pomona* beschreven en uitgegeven door het bestuur der Pomologische Vereeniging te Boskoop, by K.J.W. Ottolander, A. Koster & C. de Vos (editors). Groningen, J.B. Wolters, [1875-] 1876-1879. Chromolithograph by A.J. Wendel. Photograph: Wikipedia, G. King, 2002.

Family: Rosaceae, *Rubus chamaemorus* (**Cloudberry**). This is a slow-growing alpine or sub-arctic species of *Rubus*, producing amber-colored edible fruit. The cloudberry grows to 10 – 25 cm high. The leaves alternate between having 5 and 7 soft, handlike lobes on straight, branchless stalks. After pollination, the white (sometimes reddish-tipped) flowers form raspberry-sized berries. Encapsulating between 5 and 25 drupelets, each fruit is initially pale red, ripening into an amber colour in early autumn. Cloudberries occur naturally throughout the Northern Hemisphere mainly in mountainous areas. In Europe and Asia, they grow in the Nordic countries, the moorlands of Britain and Ireland, the Baltic states and across northern Russia east to the Pacific Ocean. Small populations are also found further south, as a botanical vestige of the Ice Ages; it is found in Germany's Weser and Elbe valleys, where it is under legal protection. In North America, cloudberries grow wild across most of Canada and Alaska and in the lower 48 states of the United States in northern Minnesota, New Hampshire, Maine and a small population on Long Island, New York. Illustration: C. A. M.

Lindman, Bilder ur Nordens Flora, Projekt Runeberg: published 1917–1926. Photograph: Wikipedia, Veli Holopainen, 2004.



Family: Rosaceae, *Rubus idaeus* (**Raspberry**). This is a commonly cultivated edible soft fruit for commercial purposes and is frequently found throughout the United Kingdom and Europe. This shrub grows to around 1.5m high with the leaves being pinnate with 3 -7 ovate leaflets. Their flowers are about 1 cm across and are white and the fruits are an aggregate of numerous drupelets. Illustration: Köhler's Medicinal Plants, 1887. Photograph: Wikipedia, Juhanson, 2004.



Family: Rosaceae, *Fragaria vesca* (**Woodland/Wild Strawberry**). This vastly edible soft fruit is common to the Northern Hemisphere (temperate Asia, Europe and North America) where it has been cultivated for its fruit for many years. A larger fruit variety is now cultivated which is a hybrid (*Fragaria x ananassa* – Garden Strawberry). This herbaceous perennial plant spreads by seed, short rhizomes and leafless stolons. The toothed leaves are thin and basal with a petiole generally 3 – 12 cm. They appear in leaflets of 3 and are sparsely hairy above. The flowers have 5 white petals with numerous pistils and 20 – 35 stamens. The five bractlets are often bilobed. The red fleshy fruit is covered with achenes. Illustration: Prof. Dr. Otto Wilhelm Thomé *Flora von Deutschland, Österreich und der Schweiz* 1885, Gera, Germany. Photograph: Wikipedia, Philip Jagenstedt, 2005.

Family: Rosaceae, *Sorbus aucuparia* (**Rowan**). They are native throughout the cool temperate regions of the northern hemisphere, with the highest species diversity in the mountains of western China and the Himalaya, where numerous apomictic microspecies occur. Rowans are mostly small deciduous trees 10 – 20 metres (30 – 67 feet) tall, though a few are shrubs. The best known species is the 'Rowan Clark' *Sorbus aucuparia*, a small tree typically 4 – 12 metres (13 – 40 feet) tall growing in a variety of habitats throughout northern Europe and in mountains in southern Europe and southwest Asia. Its berries are a favourite food for many birds and are a traditional wild-collected food in Britain and Scandinavia.The leaves are arranged alternately and are pinnate, with (8)-11– 35 leaflets. The flowers are borne in dense corymbs; each flower is creamy white and 5 – 10 mm across with five petals. The fruit is a small pome 4-8 mm diameter, bright orange or red in most species, but pink, yellow or white in some Asian species. Illustration: Prof. Dr. Otto Wilhelm Thomé *Flora von Deutschland, Österreich und der Schweiz* 1885, Gera, Germany. Photograph: Wikipedia, Krzysztof P. Jasiutowicz, 2005.

Appendix 3. Gas chromatography-Mass spectroscopy of plant oils used.

Elemi:

1 – α-Pinene (0.52%)

3 – β-Pinene (0.23%)

5 – Sabinene (5.34%)

7 – Hyrcrene (0.44%)

8 – α-Phelanohene (16.23%)

9 – α-Teyinene (0.31%)

10 – Limonene (51.54%)

11 – β-Phelandrene (2.39%)

13 – C. β-Ocimene (0.34%)

15 – γ-Teyinene (0.2%)

16 – T. β-Ocimene (0.25%)

18 – Para-Cymene (1.41%)

19 – Terpinolene (0.92%)

74 – α-Terpinene (1.64%)

99 – Methyl Eugenol (0.32%)

A – Elemol (10.9%)

B – Elemicine (4.37%)

Print Date: Wed Mar 03 13:15:47 2004 Page 1 of 2 31.5.

Title :
Run File : c:\star\data\elemi 4031001900001.run
Method File : C:\Star\methodes\essence-3800.mth
Sample ID : elemi 4031001900
A22661
Injection Date: 03/03/04 11:35 Calculation Date: 03/03/04 13:15

Operator : LABO Detector Type: 3800 (1 Volt)
Workstation: Bus Address : 44
Instrument : Varian 3800 Sample Rate : 10.00 Hz
Channel : Front = FID Run Time : 99.898 min

** Star Chromatography Workstation Version 5.51 ** 00840-7168-5CF-2180 **

Run Mode : Analysis
Peak Measurement: Peak Area
Calculation Type: Percent

Peak No.	Peak Name	Result []	Ret. Time (min)	Time Offset (min)	Area (counts)	Sep. Code	Width 1/2 (sec)	Statu Codes
1		0.52	4.355	0.000	3249	BB	3.5	
2		0.23	5.998	0.000	1415	VP	3.4	
3		5.34	6.333	0.000	33032	PB	3.4	
4		0.44	7.552	0.000	2744	BV	4.3	
5		16.23	7.785	0.000	100446	VB	5.4	
6		0.31	8.230	0.000	1906	BB	4.6	
7		51.54	9.304	0.000	319049	BV	11.3	
8		2.39	9.542	0.000	14779	VB	3.8	
9		0.34	10.789	0.000	2095	BB	4.5	
10		0.20	11.377	0.000	1241	BV	5.0	
11		0.25	11.790	0.000	1523	VB	5.0	
12		1.41	13.003	0.000	8753	VV	5.6	
13		0.92	13.813	0.000	5722	VB	5.9	
14		0.45	26.416	0.000	2794	BP	4.7	
15		0.14	27.602	0.000	855	VB	4.8	
16		0.09	30.919	0.000	570	VV	4.5	
17		0.12	32.889	0.000	723	VV	4.6	
18		0.18	33.015	0.000	1109	VV	4.6	
19		0.29	33.571	0.000	1769	VP	4.4	
20		0.11	36.442	0.000	661	PV	4.6	
21		1.64	37.985	0.000	10148	PB	4.3	
22		0.15	38.262	0.000	907	TS	0.0	
23		0.08	39.154	0.000	525	TF	0.0	
24		0.17	42.535	0.000	1027	VV	4.5	
25		0.11	47.293	0.000	664	PV	4.4	
26		0.32	50.388	0.000	1952	VV	0.0	
27		0.08	50.783	0.000	512	VB	4.4	
28		10.90	52.755	0.000	67447	PB	6.1	
29		0.15	52.976	0.000	958	TS	0.0	
30		0.16	55.616	0.000	1006	VV	5.1	
31		0.15	57.351	0.000	925	VV	4.7	
32		4.37	57.647	0.000	27076	VB	4.8	
33		0.09	73.045	0.000	557	BV	0.0	
34		0.14	80.290	0.000	878	BV	0.0	
	Totals:	100.01		0.000	619017			

Total Unidentified Counts : 619016 counts

Detected Peaks: 283 Rejected Peaks: 249 Identified Peaks: 0

Multiplier: 1 Divisor: 1 Unidentified Peak Factor: 0

Baseline Offset: 0 microVolts

Noise (used): 1 microVolts - monitored before this run

Title :
Run File : c:\star\data\elemi 4031001900001.run
Method File : C:\Star\methodes\essence-3800.mth
Sample ID : elemi 4031001900

122661

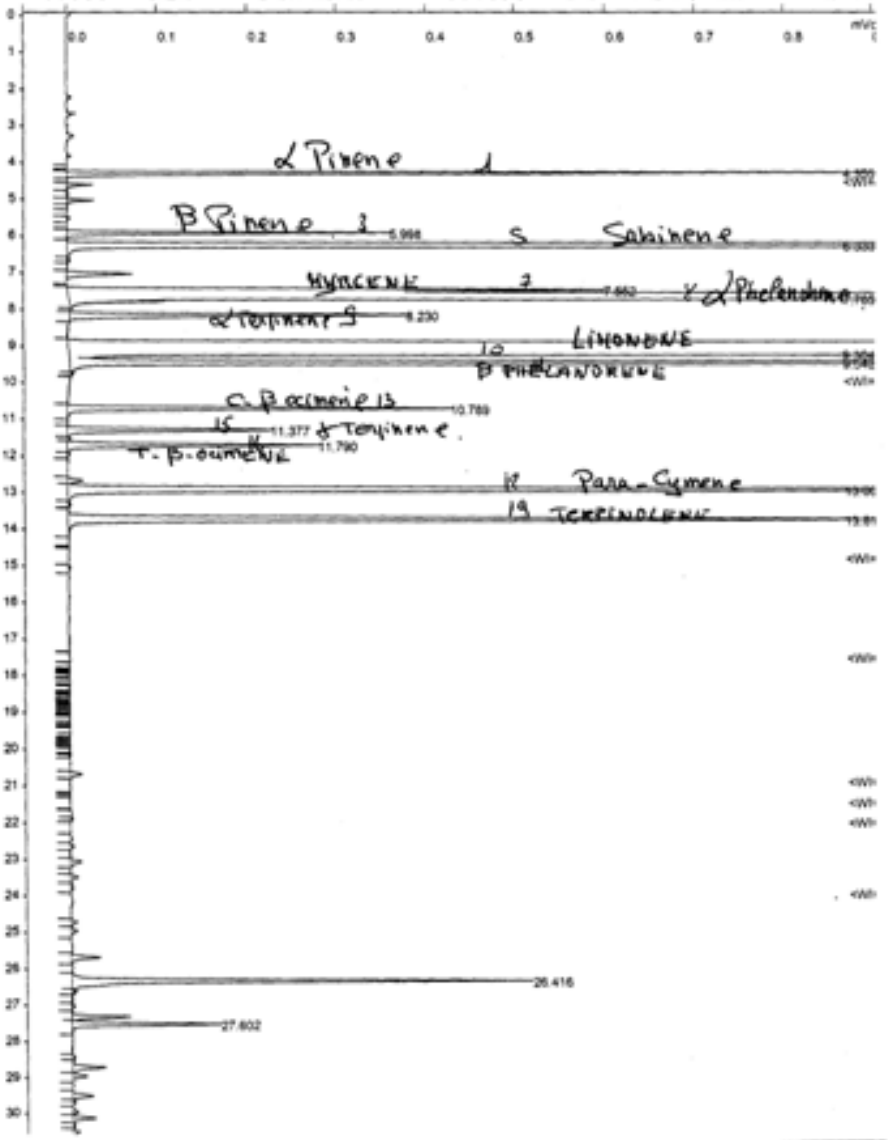
Injection Date: 03/03/04 11:35 Calculation Date: 03/03/04 13:15

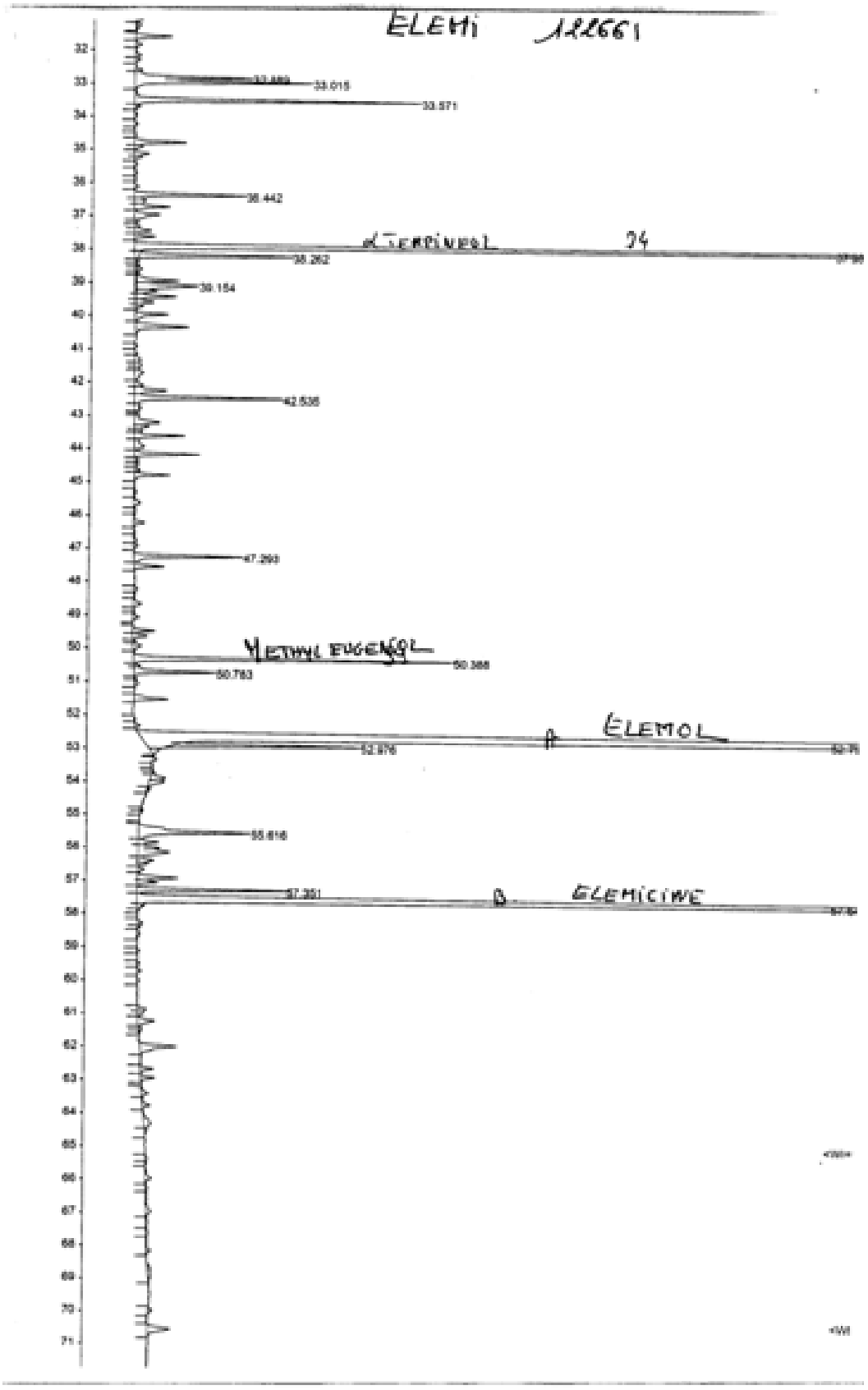
Operator : LABO
Workstation:
Instrument : Varian 3800
Channel : Front = FID

Detector Type: 3800 (1 Volt)
Bus Address : 44
Sample Rate : 10.00 Hz
Run Time : 99.898 min

** Star Chromatography Workstation Version 5.51 ** 00840-7168-5CF-2180 **

Chart Speed = 0.70 cm/min Attenuation = 4 Zero Offset = 5%
Start Time = 0.000 min End Time = 99.898 min Min / Tick = 1.00




ELEMI
α-TERPINEOL
γ-METHYL EUGENOL
ELEMOL
ELEMICINE

Myrtle:

1 – α-Pinene (51.72%)

A – Unknown (0.39%)

3 – β-Pinene (0.54%)

6 – Δ,3-Carene (0.66%)

9 – α-Teyinene (0.46%)

10 – Limonene (8.34%)

12 – 1,8-Cineole (22.25%)

15 – γ-Teyinene (0.29%)

16 – Unknown (0.233%)

18 – Para-Cymene (1.83%)

19 – Teyinolene (0.89%)

48 – Linalool (3.67%)

51 – Actelinalyle (0.6%)

56 – β-Caryophyllene (0.7%)

59 – Teyinene (0.31%)

MC – Methyl chavicol (0.13%)

72 – Unknown (0.41%)

74 – γ-Teyineol (1.91%)

85 – Acte Geranyle (1.96%)

92 – Geraniol (0.3 / 0.1%)

99 – Unknown (0.41%)

: c:\star\data\2005\essence-3800 2005\myrte5071046400.run
File : C:\Star\methodes\essence-3800.mth
ID : myrte5071046400

A16471 . 116472 .

on Date: 01/08/05 12:06 Calculation Date: 01/08/05 13:46

: LABO
ion:
ent : Varian 3800
: Front = FID

Detector Type: 3800 (1 Volt)
Bus Address : 44
Sample Rate : 10.00 Hz
Run Time : 99.898 min

Chromatography Workstation Version 5.51 ** 00840-7168-SCF-2180 **

: Analysis
asurement: Peak Area
ion Type: Percent

Peak Name	Result (%)		Ret. Time (min)	Time Offset (min)	Area (counts)	Sep. Code	Width 1/2 (sec)	Status Codes
	51.72	1	4.409	0.000	276894	BB	3.7	
	0.05		5.081	0.000	272	BB	2.5	
	0.39	A	5.506	0.000	2071	BB	2.4	
	0.54	3	5.952	0.000	2881	BB	2.9	
	0.07		6.258	0.000	382	BV	3.0	
	0.66	6	7.041	0.000	3509	BB	3.3	
	0.19		7.451	0.000	1021	BV	3.4	
	0.15		7.585	0.000	797	VV	3.6	
	0.46	9	7.990	0.000	2467	VV	3.8	
	0.05		8.111	0.000	277	VB	4.8	
	8.34	10	8.954	0.000	44654	BB	5.3	
	22.25	12	9.555	0.000	119104	BB	7.5	
	0.29	15	11.202	0.000	1555	BB	5.0	
	0.33	16	11.605	0.000	1785	BB	5.1	
	1.83	18	12.877	0.000	9821	BB	5.6	
	0.89	19	13.599	0.000	4773	BB	5.9	
	0.06		20.350	0.000	335	BB	5.9	
	0.07		24.711	0.000	382	BB	5.5	
	3.67	48	31.297	0.000	19623	BV	4.5	
	0.60	54	31.520	0.000	3189	VB	4.6	
	0.11		32.625	0.000	596	PV	4.8	
	0.70	56	32.836	0.000	3770	VB	4.7	
	0.31	58	33.698	0.000	1659	BB	4.5	
	0.09		36.136	0.000	484	BV	4.9	
	0.26		36.274	0.000	1386	VB	4.9	
	0.13	MC	36.833	0.000	718	BV	4.6	
	0.07		37.275	0.000	377	BB	4.5	
	0.41	72	37.863	0.000	2198	VV	4.6	
	1.91	74	38.135	0.000	10202	VB	4.4	
	0.06		38.420	0.000	325	TS	0.0	
	0.12		39.285	0.000	620	BB	4.6	
	0.05		39.633	0.000	285	BB	5.1	
	1.96	85	40.600	0.000	10484	BB	4.3	
	0.14		43.089	0.000	750	BB	4.7	
	0.30	92	44.315	0.000	1626	BV	4.3	
	0.11		44.434	0.000	592	VB	5.3	
	0.05		47.715	0.000	269	BB	0.0	
	0.13		49.180	0.000	691	VB	4.9	
	0.41	99	50.496	0.000	2176	BB	4.5	
	0.07		51.471	0.000	356	VB	4.7	
tals:	100.00			0.000	535356			

Identified Counts : 535355 counts

Peaks: 68 Rejected Peaks: 28 Identified Peaks: 40

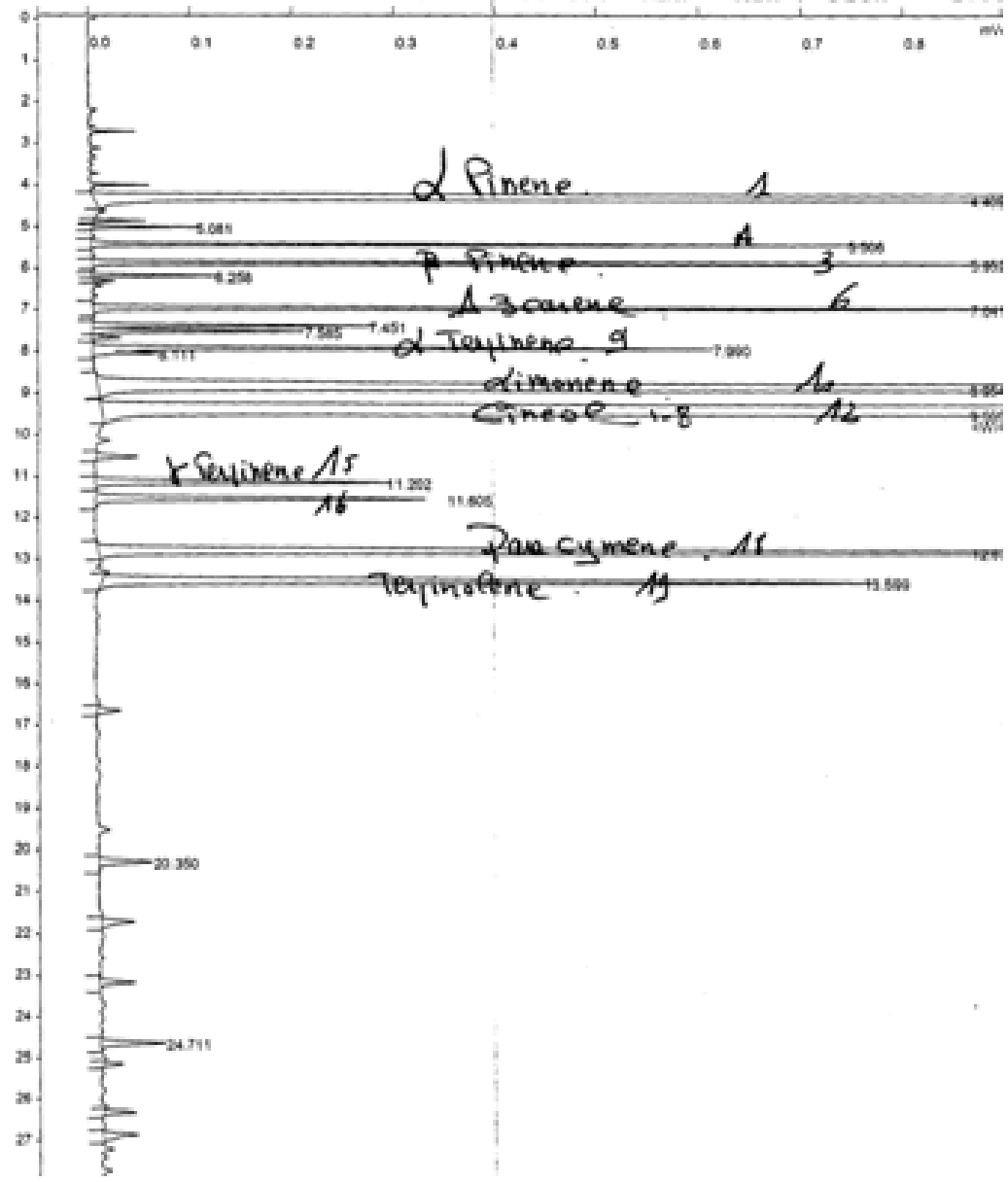
: c:\star\data\2005\essence-3800 2005\myrte5071046400.run
: C:\Star\methodes\essence-3800.mth
: myrte5071046400

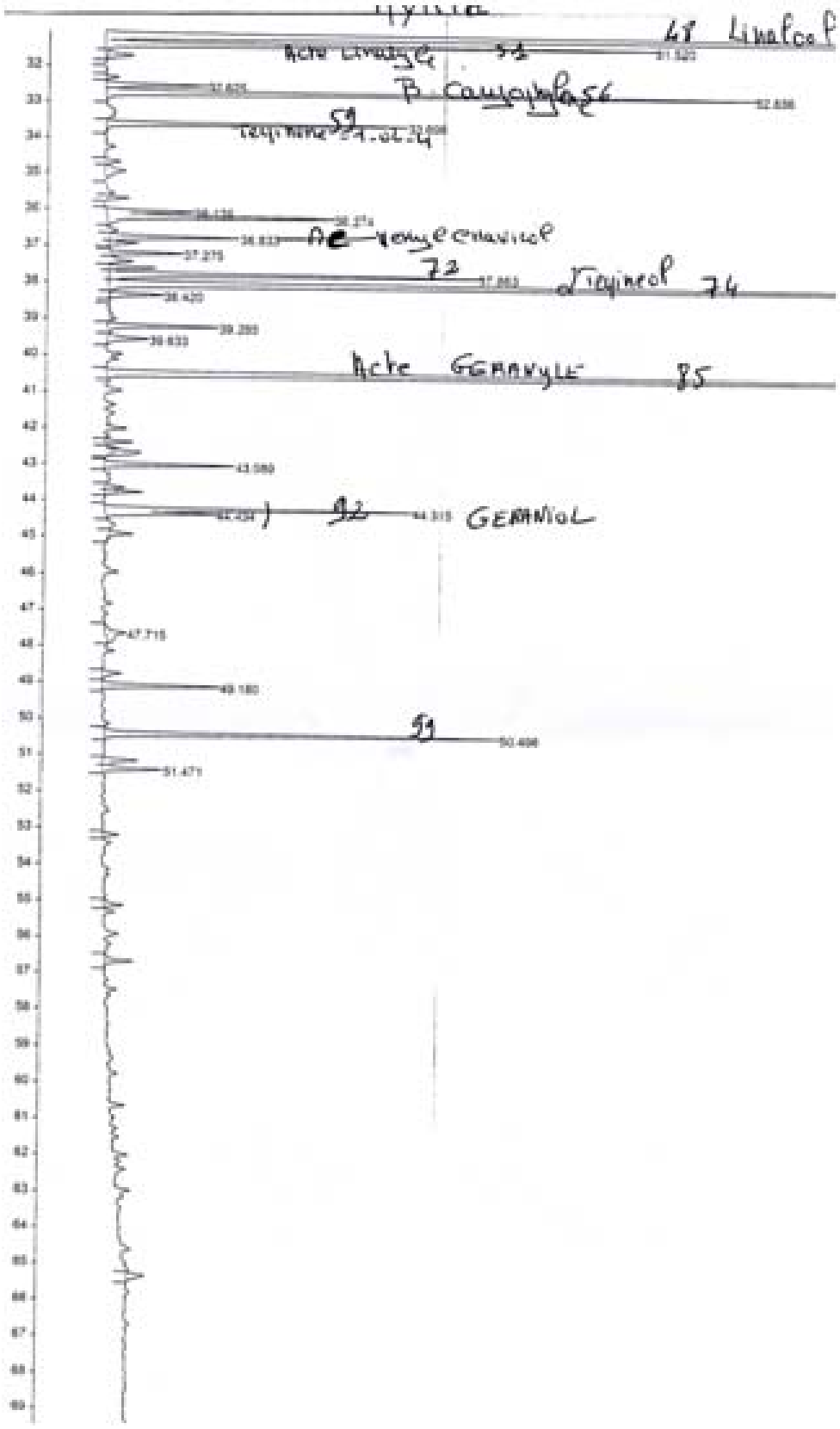
ate: 01/08/05 12:06 Calculation Date: 01/08/05 13:46

: LABO Detector Type: 3800 (1 Volt)
: Bus Address : 44
: Varian 3800 Sample Rate : 10.00 Hz
: Front = FID Run Time : 99.898 min

omatography Workstation Version 5.51 ** 00840-7169-5CF-2180 **

= 0.70 cm/min Attenuation = 4 Zero Offset = 5%
= 0.000 min End Time = 99.898 min Min / Tick = 1.0




Linalool
Acte Linalyle
B. caryophyllene
Terpinene-4-ol
Acte Geranyle
Geraniol

Myrrh:

1 – D-Elemene (0.71%)

4 – Unknown (0.07%)

9 – Unknown (0.23%)

10 – β-Elemene (3.63%)

13 – Elemene (1.14%)

18 – D-Germacrene (0.98%)

23 – Unknown (0.8%)

26 – β-Germacrene (2.18%)

28 – Curezene (26.39%)

32 – Unknown (1.5%)

37 – Furano (Endem – 1,3 diene) (31.97%)

38 – Unknown (7.49%)

44 – Unknown (6.75%)

int Date: Wed Jul 20 12:22:31 2005 Page 1 of 3

tle :
n File : c:\star\data\2005\essence-3800 2005\myrrhe 5071017500.run
thod File : C:\Star\methodes\essence-3800.mth
mple ID : myrrhe 5071017500

SAP 122734

jection Date: 20/07/05 10:42 Calculation Date: 20/07/05 12:22

erator : LABO
rkstation:
strument : Varian 3800
annel : Front = FID

Detector Type: 3800 (1 Volt)
Bus Address : 44
Sample Rate : 10.00 Hz
Run Time : 99.898 min

Star Chromatography Workstation Version 5.51 ** 00840-7168-5CF-2180 **

n Mode : Analysis
ak Measurement: Peak Area
lculation Type: Percent

Peak No.	Peak Name	Result ()	Ret. Time (min)	Time Offset (min)	Area (counts)	Sep. Code	Width 1/2 (sec)	Status Codes
1		0.10	11.740	0.000	578	BB	5.2	
2		0.71	26.398	0.000	3911	PB	4.9	
3		0.07	27.408	0.000	393	BB	4.9	
4		0.21	28.901	0.000	1177	BV	4.9	
5		0.23	31.829	0.000	1286	BV	6.8	
6		0.07	32.292	0.000	359	VP	4.7	
7		0.10	32.659	0.000	555	PV	6.3	
8		3.63	32.890	0.000	19979	VB	5.3	
9		1.14	35.148	0.000	6277	VP	4.6	
10		0.09	36.146	0.000	479	PV	0.0	
11		0.20	36.410	0.000	1082	VV	4.7	
12		0.15	36.713	0.000	828	VV	7.1	
13		0.16	37.316	0.000	866	VV	4.7	
14		0.05	37.565	0.000	302	VV	8.3	
15		0.98	38.232	0.000	5407	VV	0.0	
16		0.67	38.613	0.000	3704	VV	5.2	
17		0.62	38.847	0.000	3404	VV	4.8	
18		0.22	39.195	0.000	1192	VV	4.8	
19		0.80	40.365	0.000	4416	VP	5.9	
20		0.32	41.159	0.000	1764	PV	5.3	
21		2.18	43.273	0.000	12007	VP	5.1	
22		26.39	45.292	0.000	145401	BP	8.7	
23		0.05	46.769	0.000	261	TF	0.0	
24		0.08	47.297	0.000	432	TF	0.0	
25		0.11	47.827	0.000	595	TF	0.0	
26		0.12	48.107	0.000	661	TF	0.0	
27		0.18	48.377	0.000	974	TF	0.0	
28		0.10	48.620	0.000	554	TF	0.0	
29		0.06	48.734	0.000	342	TF	0.0	
30		0.08	48.893	0.000	450	TF	0.0	
31		0.07	48.983	0.000	368	TF	0.0	
32		0.05	49.057	0.000	298	TF	0.0	
33		0.42	49.349	0.000	2300	TF	0.0	
34		0.11	49.667	0.000	587	TF	0.0	
35		0.20	49.766	0.000	1078	TF	0.0	
36		0.14	50.023	0.000	789	TF	0.0	
37		0.40	50.335	0.000	2217	TF	0.0	
38		0.16	50.595	0.000	903	TF	0.0	
39		0.10	50.743	0.000	563	TF	0.0	
40		0.20	50.854	0.000	1116	TF	0.0	
41		1.50	51.135	0.000	8265	TF	0.0	
42		0.06	51.312	0.000	337	TF	0.0	
43		0.23	51.391	0.000	1252	TF	0.0	
44		0.74	51.800	0.000	4098	TF	0.0	
45		0.31	52.023	0.000	1691	TF	0.0	
46		0.14	52.264	0.000	770	mm	0.0	

MYRRHE 122734 27 (2)

Print Date: Wed Jul 20 12:22:31 2005 Page 2 of 3

47	0.06	52.496	0.000	312	TF	0.0	
48	0.05	52.646	0.000	281	TF	0.0	
49	0.08	52.761	0.000	422	TF	0.0	
50	0.27	52.938	0.000	1466	TF	0.0	
51	0.15	53.226	0.000	851	TF	0.0	
52	0.25	53.545	0.000	1372	TF	0.0	
53	31.97 37	54.569	0.000	176124	PV	10.1	
54	7.49 38	54.696	0.000	41249	VB	5.1	
55	0.13	54.984	0.000	724	TF	0.0	
56	0.05	55.136	0.000	273	TF	0.0	
57	0.07	55.688	0.000	405	BV	6.9	
58	1.57	55.900	0.000	8673	VV	4.8	
59	0.16	56.294	0.000	877	VV	5.5	
60	0.66	56.430	0.000	3615	VV	4.7	
61	0.15	56.614	0.000	839	VV	5.0	
62	0.08	56.801	0.000	427	VV	6.8	
63	0.05	57.169	0.000	299	VV	0.0	
64	0.06	57.455	0.000	312	VV	0.0	
65	0.11	57.566	0.000	602	VV	7.7	
66	0.08	57.758	0.000	416	VV	7.0	
67	0.24	57.938	0.000	1335	VV	15.4	
68	6.75 44	58.137	0.000	37206	VB	5.5	
69	0.47	58.686	0.000	2563	TF	0.0	
70	0.05	59.009	0.000	293	TF	0.0	
71	0.05	60.014	0.000	292	VB	4.5	
72	0.08	62.262	0.000	448	BV	4.9	
73	0.05	62.444	0.000	296	VV	5.4	
74	0.06	63.233	0.000	345	VV	5.6	
75	0.06	63.397	0.000	313	VV	5.8	
76	0.06	64.081	0.000	349	VV	8.1	
77	0.16	64.292	0.000	861	VV	5.3	
78	0.06	64.500	0.000	314	VV	8.3	
79	0.08	64.971	0.000	465	VV	5.9	
80	0.19	65.669	0.000	1072	BV	6.8	
81	0.05	66.061	0.000	289	VV	7.3	
82	0.07	66.453	0.000	369	VV	12.1	
83	0.05	66.877	0.000	268	VV	0.0	
84	0.06	67.190	0.000	338	VV	0.0	
85	1.15	67.933	0.000	6317	VV	8.8	
86	0.37	68.543	0.000	2015	VV	0.0	
87	0.60	68.820	0.000	3331	VV	0.0	
88	0.29	69.339	0.000	1591	VV	0.0	
89	0.09	69.707	0.000	519	VV	16.5	
90	0.22	70.101	0.000	1187	VB	6.9	
91	0.05	72.493	0.000	271	BV	8.4	
92	0.19	77.256	0.000	1039	VB	9.5	
93	0.05	80.819	0.000	301	BB	13.6	
94	0.07	82.236	0.000	375	BV	15.7	
95	0.07	84.993	0.000	390	BV	15.2	
96	0.07	88.200	0.000	373	BB	11.2	
---	---	---	---	---	---	---	---
Totals:	100.00		0.000	550941			

tal Unidentified Counts : 550944 counts

tected Peaks: 189 Rejected Peaks: 93 Identified Peaks: 0

ltiplier: 1 Divisor: 1 Unidentified Peak Factor: 0

seline Offset: 80 microVolts

ise (used): 2 microVolts - monitored before this run

el: 14 Injection Number: 1 Volume: 0.2 uL Position: 1

ginal Notes:

122734

27

: c:\star\data\2005\essence-3800 2005\myrrhe 5071017500.run
ile : C:\Star\methodes\essence-3800.mth
D : myrrhe 5071017500

122734

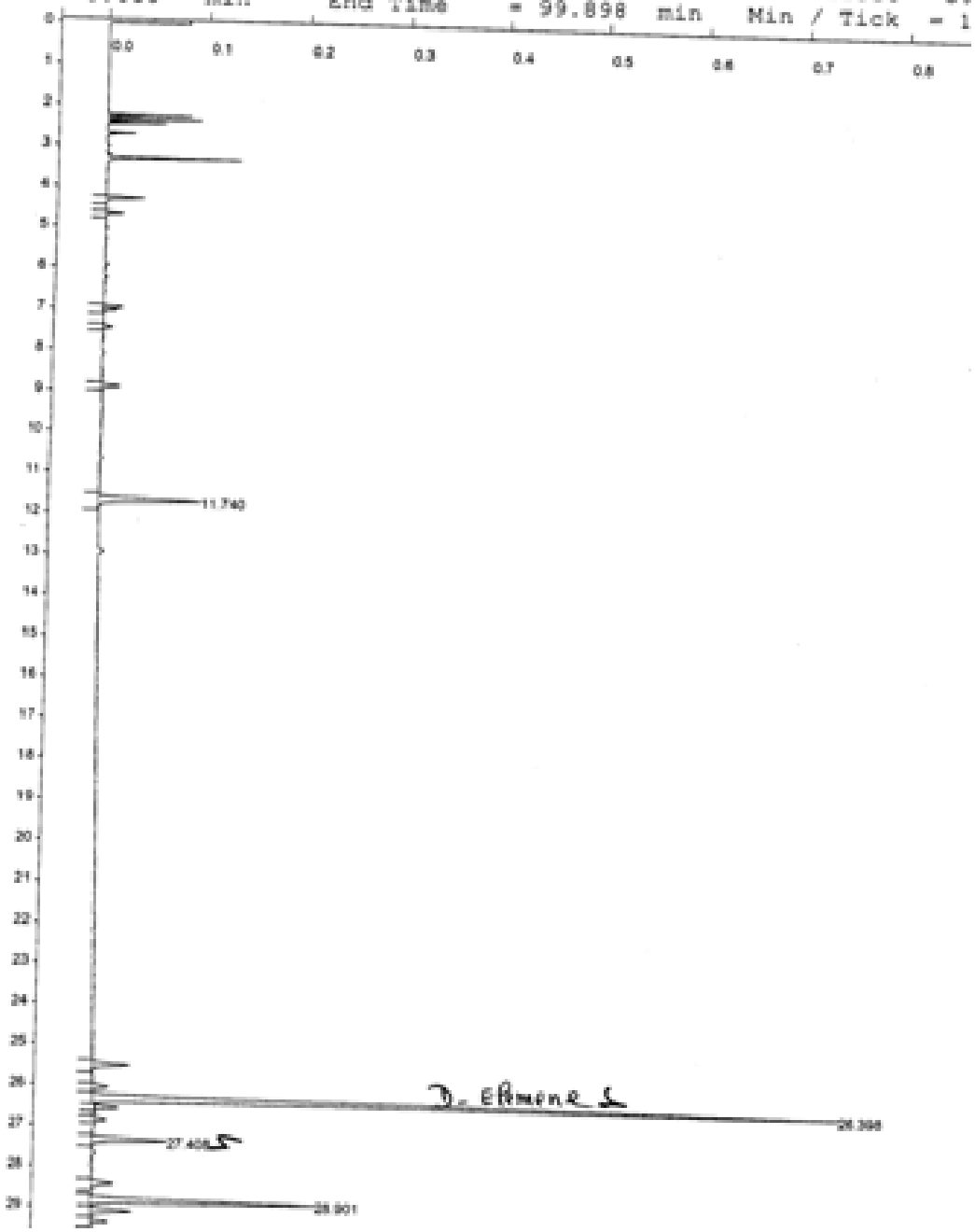
n Date: 20/07/05 10:42 Calculation Date: 20/07/05 12:22

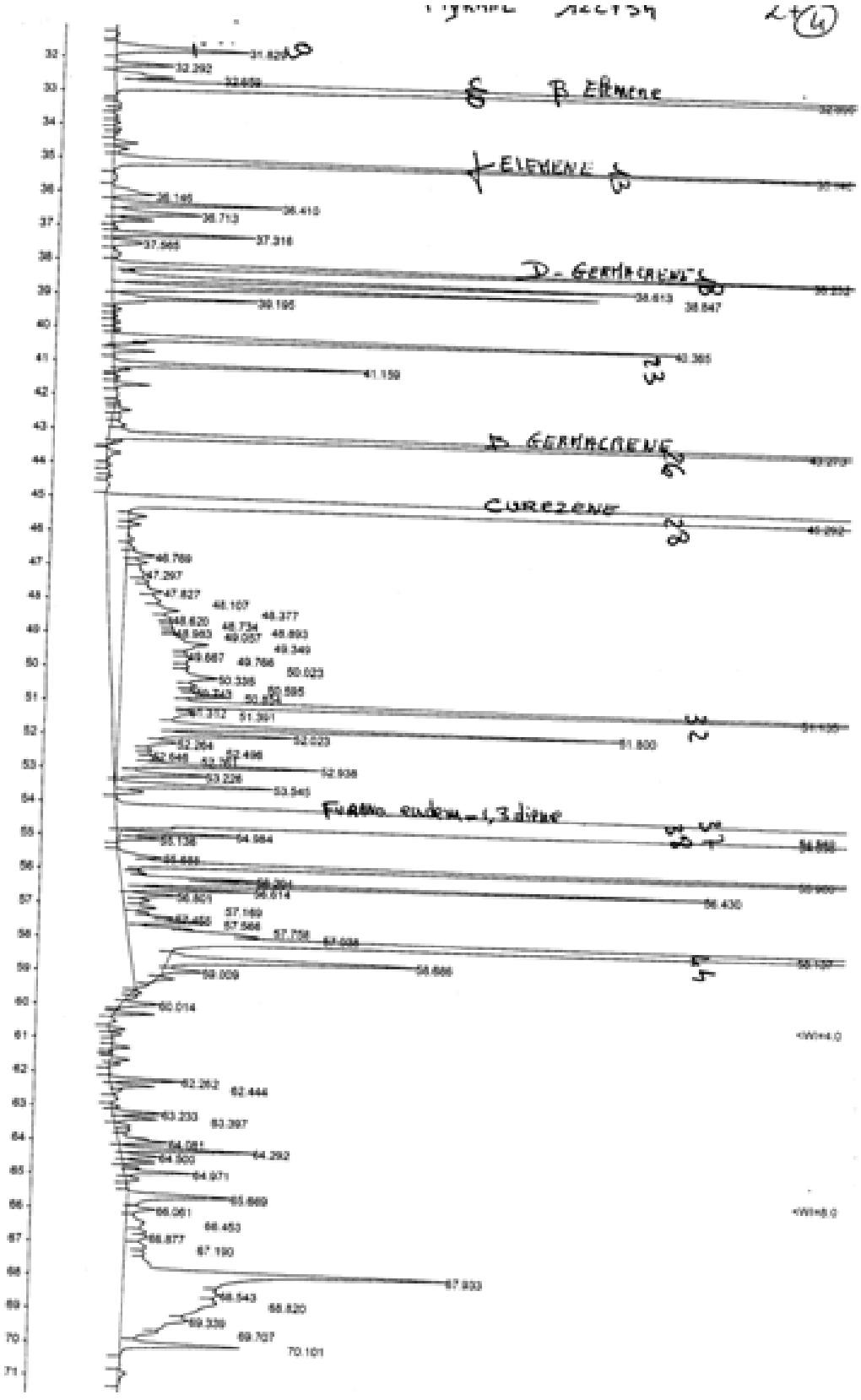
: LABO
ion:
nt : Varian 3800
: Front = FID

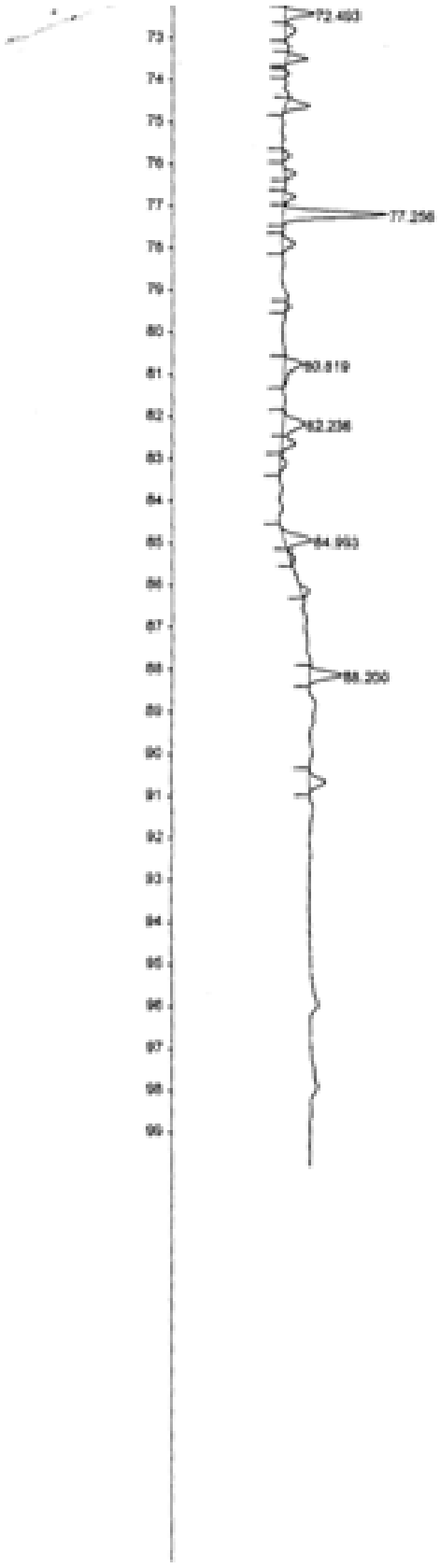
Detector Type: 3800 (1 Volt)
Bus Address : 44
Sample Rate : 10.00 Hz
Run Time : 99.898 min

Chromatography Workstation Version 5.51 ** 00840-7168-5CF-2180 **

eed = 0.70 cm/min Attenuation = 4 Zero Offset = 5%
ne = 0.000 min End Time = 99.898 min Min / Tick = 1




β ELEMENE
ELEMENE
D-GERMACRENE
GERMACRENE
CURCUMENE
FURANO eudesm-1,3 diene


72.493
77.256
80.819
82.236
84.953
88.200

Sweet Fennel:
1 – Fenchone
2 – *trans*-anethole

GAS CHROMATOGRAPHY REPORT

Sample name : S251T SWT FENNEL

Injected on : 13/9/05 3:53:56 AM
Acq. Method : LONG.M
Data File : C:\HPCHEM\2\DATA\C050912\013F1501.D

#	Height	Area	Meas. Ret.	Area %	Compound Name
1	190	406.92	5.88	3.06	
2	25	58.96	7.41	0.44	
3	31	74.35	8.47	0.56	
4	258	605.27	8.65	4.55	
5	98	234.73	9.48	1.76	
6	26	63.22	9.76	0.48	
7	16	42.86	10.70	0.32	
8	41	102.24	11.44	0.77	
9	505	1870.72	15.49	14.06	–1
10	10	29.46	18.38	0.22	
11	213	655.98	23.97	4.93	
12	10	31.58	26.59	0.24	
13	1426	9093.43	28.83	68.33	–2
14	10	38.25	34.37	0.29	

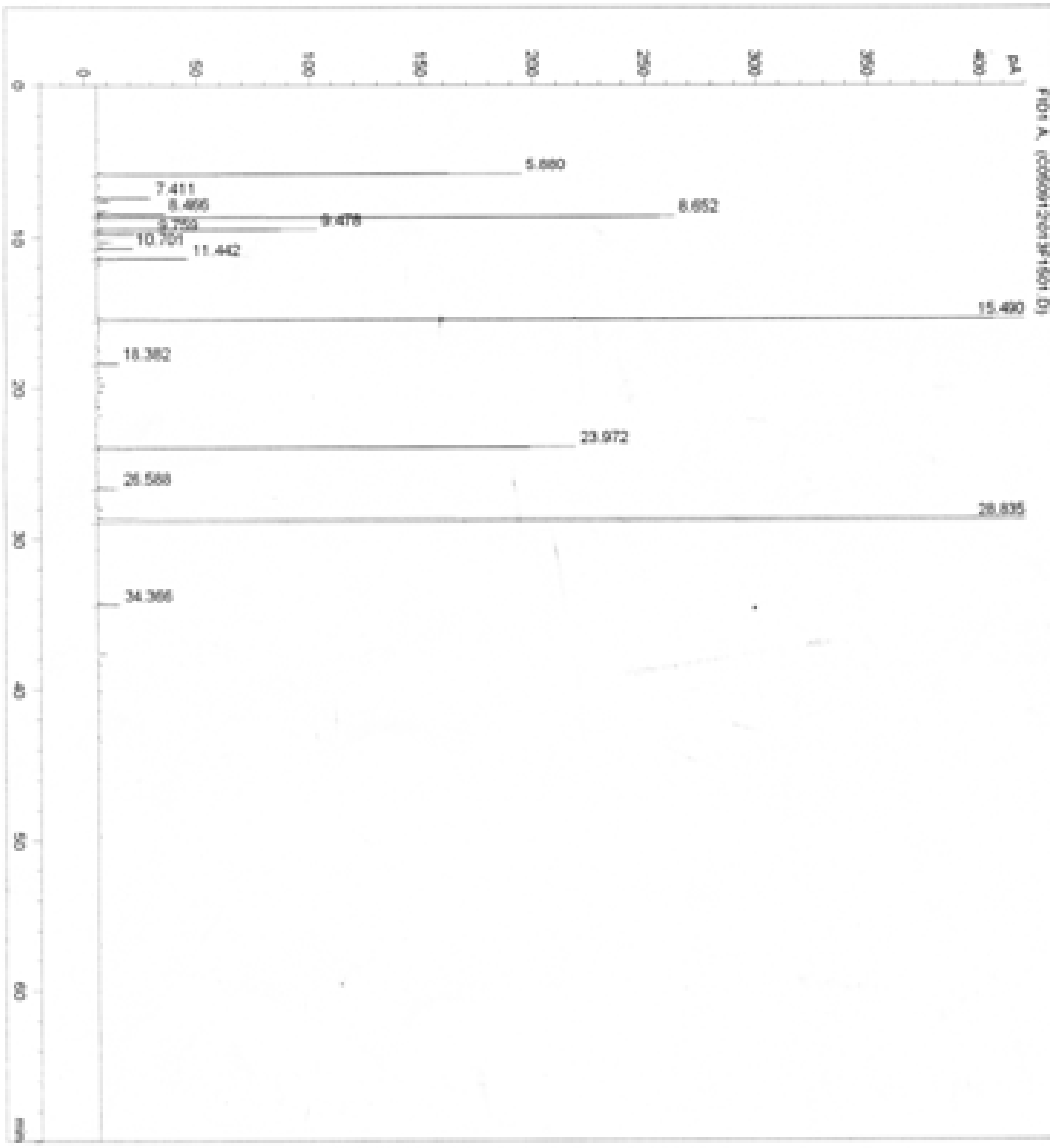
*** End of Report ***

GAS CHROMATOGRAPHY REPORT

COLANGE BRAND

Sample name : S251T SWT FENNEL

Injected on : 13/9/05 3:53:56 AM
Acquisition method : LONG.M
Data File : C:\HPCHEM\2\DATA\C050912\013F1501.D
Sample info : SWEET FENNEL OIL



Geranium:
1 – Citronellol

Sample name : S258T GERANIUM

Injected on : 13/9/05 2:36:06 AM
Acq. Method : LONG.M
Data File : C:\HPCHEM\2\DATA\C050912\012F1401.D

#	Height	Area	Meas. Ret.	Area %	Compound Name
1	30	63.54	5.88	0.60	
2	66	173.39	13.88	1.64	
3	26	69.17	14.36	0.65	
4	43	131.64	17.57	1.24	
5	217	743.59	18.49	7.01	
6	38	113.28	19.29	1.07	
7	149	409.11	19.81	3.86	
8	17	56.82	21.49	0.54	
9	42	134.06	21.81	1.26	
10	186	658.39	22.05	6.21	
11	309	1104.04	22.32	10.41	
12	19	64.52	22.56	0.61	
13	12	39.52	23.33	0.37	
14	14	37.49	23.51	0.35	
15	13	54.70	24.06	0.52	
16	12	44.75	24.65	0.42	
17	31	100.60	24.82	0.95	
18	80	229.56	24.90	2.16	
19	48	162.92	25.28	1.54	
20	29	111.59	25.47	1.05	
21	12	39.03	25.62	0.37	
22	14	52.12	25.82	0.49	
23	826	4210.33	26.64	39.70	–1
24	14	39.00	27.61	0.37	
25	38	109.27	27.68	1.03	
26	9	39.75	27.99	0.37	
27	27	81.70	28.22	0.77	
28	290	981.29	28.94	9.25	
29	8	41.28	29.82	0.39	
30	30	83.24	30.36	0.78	
31	8	23.70	31.78	0.22	
32	30	87.73	33.59	0.83	
33	13	40.85	35.86	0.39	
34	54	198.44	36.27	1.87	
35	22	74.08	38.49	0.70	

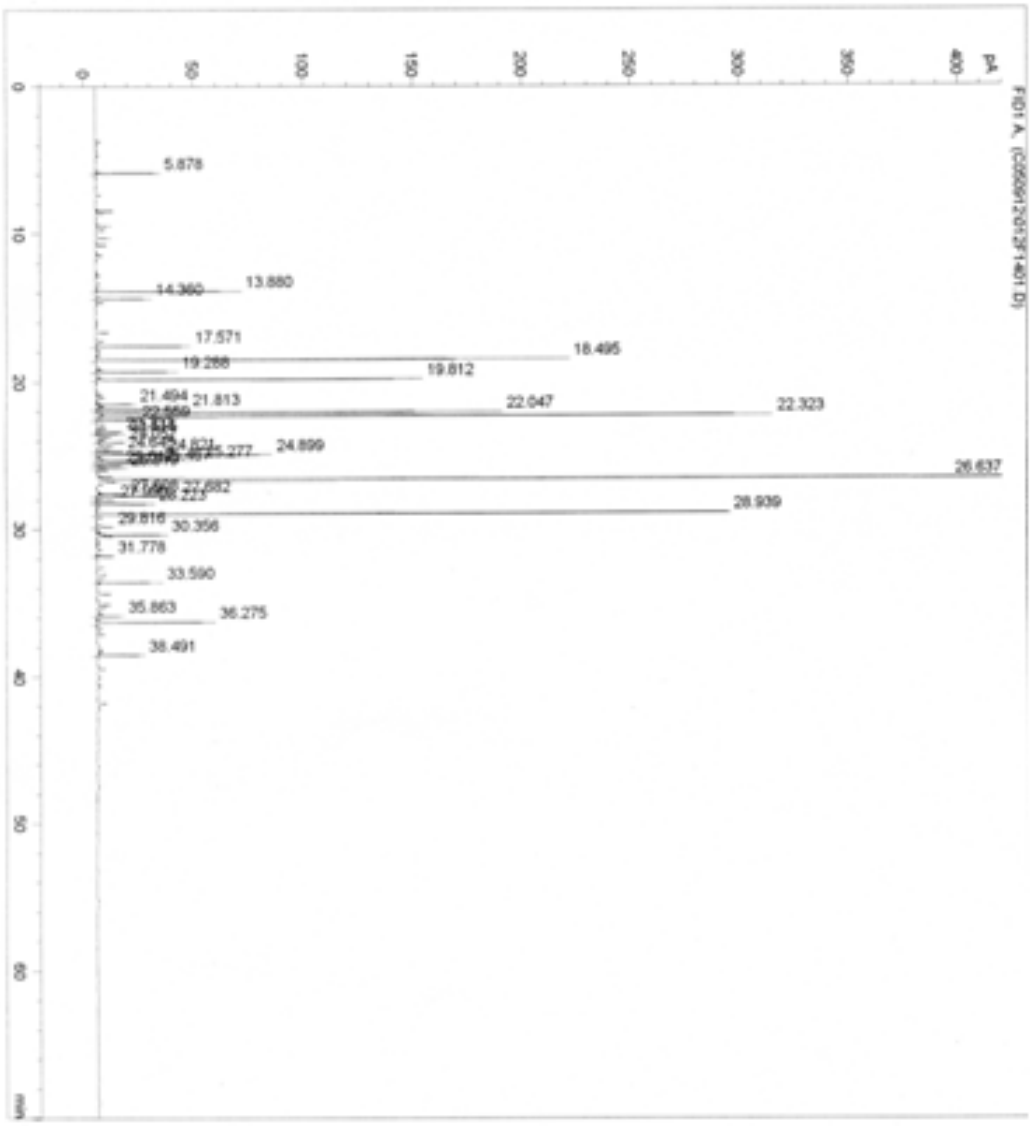
*** End of Report ***

GAS CHROMATOGRAPHY REPORT

COLANCO BRAND

Sample name : S258T GERANIUM

Injected on : 13/9/05 2:36:06 AM
Acquisition method : LONG.M
Data File : C:\HPCHEM\2\DATA\C050912\012F1401.D
Sample info : GERANIUM OIL



Palmarosa:

1 – Geraniol

GAS CHROMATOGRAPHY REPORT

Sample name : S253T PALMAROSA

Injected on : 13/9/05 5:11:37 AM
Acq. Method : LONG.M
Data File : C:\HPCHEM\2\DATA\C050912\014F1601.D

#	Height	Area	Meas. Ret.	Area %	Compound Name
1	12	25.94	8.46	0.22	
2	25	59.17	10.27	0.50	
3	97	227.89	10.77	1.91	
4	157	426.78	19.81	3.57	
5	95	302.83	21.81	2.53	
6	14	39.62	25.81	0.33	
7	300	1059.68	26.42	8.87	
8	1442	9563.31	29.07	80.02	— 1
9	10	27.85	34.24	0.23	
10	29	86.74	35.61	0.73	
11	36	131.57	42.27	1.10	

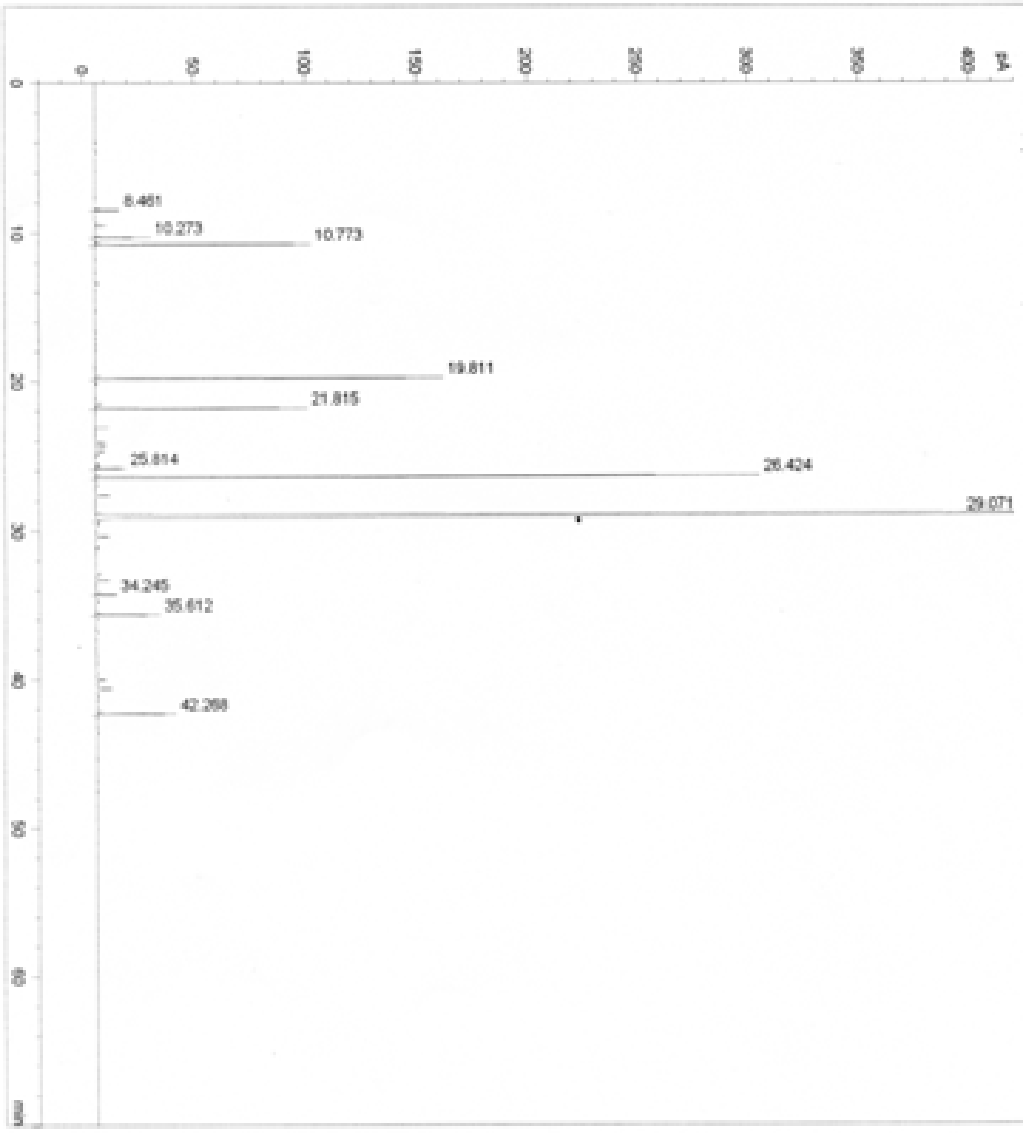
*** End of Report ***

GAS CHROMATOGRAPHY REPORT

COLANGE BRAND

Sample name : S253T PALMAROSA

Injected on : 13/9/05 5:11:37 AM
Acquisition method : LONG.M
Data File : C:\HPCHEM\2\DATA\C050912\014F1601.
Sample info : PALMAROSA OIL



Appendix 4. Keister modification of TYI-S-33 medium

K_2HPO_4	2 g (BDH Ltd., UK)
KH_2PO_4	1.2 g (BDH)
Casein Digest peptone	40 g (BD-Difco, USA)
Yeast Extract	20 g (BD-Difco)
Glucose	20 g (BDH)
NaCl	4 g (BDH)
Cysteine HCl Monohydrate	4 g (Gibco-BRL)
L Ascorbic acid	400 mg (Sigma-Aldrich)
Ferric Ammonium Citrate	45.6 mg (Sigma-Aldrich)
Dehydrated Bovine Bile	2 g (Sigma-Aldrich)

Dissolve above in 1 L distilled water and adjust pH to 7.0 with 1M Sodium hydroxide (NaOH). Adjust the final volume to 1800 ml with distilled water and then filter with a Whatman No. 1 filter paper or equivalent. Dispense into sterile 100 ml bottles after further filtering with a peristaltic pump and a 0.45 μm membrane filter (Sartorius, Germany) and store at -20^{o}C.

When culturing parasites thaw the required volume of basic medium and add 10% heat inactivated bovine (or equine) serum and 200 μl / 100 ml Gentamicin sulphate (10 mg ml^{-1} stock; Gibco-BRL) and filter with a 0.2 μm membrane filter (Sartorius).

Sterile complete medium can be stored at 4^{o}C for 2 weeks. This is often dispensed into 16 x 110 mm flat sided culture tubes, which are canted at a 5° angle from the horizontal, filled to 90 – 95% of its capacity.

Appendix 5. SDS-Polyacrylamide gel electrophoresis

Preparation of reagents

1. **Acrylamide/Bis (30% T, 2.67% C) (Solution A)**
   29.2 g acrylamide (Sigma-Aldrich, UK)
   0.8 g N'N'-bis-methylene-acrylamide (Sigma-Aldrich)
   Make to 100 ml with deionised water. Filter and store at 4 °C in the dark (30 days maximum.)

2. **10% (w/v) Sodium dodecylsulphate (SDS)**
   Dissolve 1 g SDS (Sigma-Aldrich) in 90 ml water with gentle stirring and bring to 10 ml with deionised water. Store at room temperature.

3. **1.5 M Tris-HCl, pH 8.8 buffer solution (Solution B)**
   18.15 g Tris base (Sigma-Aldrich)
   80 ml deionised water
   Adjust to pH 8.8 with 6 N HCl and bring the total volume to 100 ml with deionised water and store at 4 °C.

4. **0.5 M Tris-HCl, pH 6.8 buffer solution (Solution C)**
   6 g Tris base (Sigma-Aldrich)
   60 ml deionised water
   Adjust to pH 6.8 with 6 N HCl and bring the total volume to 100 ml with deionised water and store at 4 °C.

5. **Sample Buffer**
   3.55 ml deionised water
   1.25 ml 0.5 M Tris-HCl, pH 6.8 (Solution C)
   2.5 ml Glycerol (BDH)
   2.0 ml 10% (w/v) SDS
   0.2 ml 0.5% (w/v) Bromophenol blue (BDH)
   0.5 ml 2-Mercaptoethanol (BDH)
   10 ml total volume.
   Store at room temperature.

6. **10x Electrode (Running) Buffer, pH 8.3 (makes 1 L)**
   30.3 g Tris base
   144.0 g Glycine (Sigma-Aldrich)
   10.0 g SDS
   Dissolve and bring total volume up to 1,000 ml with deionised water. Do not adjust pH with acid or base. Store at 4 °C. If precipitation occurs, warm to room temperature before use. Mix thoroughly before use.

7. **10% Ammonium persulphate (APS) (made fresh daily)**
   100 mg APS (Sigma-Aldrich)
   Dissolved in 1 ml of deionised water.

8. Fixative solution

25 ml 100 % Methanol (BDH)
7 ml Glacial Acetic Acid (BDH)
75 ml deionised water
Prepare a 25 % (v/v) Methanol solution by combining the above volumes of methanol and water and then remove 7 ml of this. Replace the removed volume with 7 ml glacial acetic acid. This fixative solution can be re-used several times. Store at room temperature.

9. Colloidal blue G staining solution

40 ml 1x Colloidal blue G solution (Sigma-Aldrich)
10 ml 100 % Methanol
Prepare a 1x working solution of colloidal blue G as per the manufacturer's instructions and store at 4°C. Ensure that the solution is thoroughly mixed before removing a 40 ml aliquot and to this add 10 ml 100% methanol. Mix well before pouring onto gel and prepare this solution immediately before use.

10. Acetic Acid Destain

25 ml 100 % Methanol (BDH)
10 ml Glacial Acetic Acid (BDH)
75 ml deionised water
Prepare a 25 % (v/v) Methanol solution by combining the above volumes of methanol and water and then remove 10 ml of this. Replace the removed volume with 10 ml glacial acetic acid. Store at room temperature.

11. Methanol Destain

25 ml 100 % Methanol (BDH)
75 ml deionised water
Prepare a 25 % (v/v) Methanol solution by combining the above volumes of methanol and water. Store at room temperature.

Casting of Polyacrylamide gels (Bio-Rad Mini-Protean 3 system)

1. Place the Casting Frame upright with the pressure cams in the open position and facing forward on a flat surface.

2. Place a Short Plate on top of the Spacer plate.

3. Orient the Spacer Plate so that the labelling is "up". Slide the 2 glass plates into the Casting Frame, keeping the Short Plate facing the front of the frame (side with pressure cams).

4. Ensure both plates are flush on a level surface and labelling on the Spacer Plate is oriented correctly. Leaking may occur if the plates are misaligned or oriented incorrectly.

5. When the glass plates are in place, engage the pressure cams to secure the glass cassette sandwich in the Casting Frame. Check that both plates are flush at the bottom.

6. Engage the spring loaded lever and place the gel cassette assembly on the grey casting stand gasket. Insure the horizontal ribs on the back of the Casting Frame are flush against the face of the Casting Stand and the glass plates are perpendicular to the level surface. The lever pushes the Spacer Plate down against the grey rubber gasket. Mark out level of separating gel required (~ 5mm lower than the base level of the comb).

7. Prepare the separating gel (12% Acrylamide gel). The volumes given below is enough to prepare 2 0.75 mm thick gels:

   3.4 ml Deionised water
   4.0 ml Solution A
   2.5 ml Solution B
   0.1 ml 10% SDS

8. Degas under vacuum for minimum of 15 min in a conical flask with side arm to remove oxygen which inhibits the polymerisation of acrylamide.

9. Immediately prior to pouring the gel, add the catalyst:

   50 µl 10% APS
   10 µl N,N,N', N'-Tetramethylethylenediamine (TEMED; Sigma-Aldrich)

10. Carefully pour the separating gel in between the glass plates and apply a thin layer of isopropanol on top of each gel before it sets to remove air bubbles. The gel should be left for 30 min to allow it to fully polymerise.

11. During this time, prepare the stacking gel (4% acrylamide):

   6.1 ml Deionised water
   1.5 ml Solution A
   2.5 ml Solution B
   0.1 ml 10% SDS

12. Degas under vacuum for minimum of 15 min in a conical flask with side arm.

13. Remove the isopropanol by diluting and rinsing it with deionised water. Blot off the remaining water, using dry filter paper.

14. Immediately prior to pouring the stacking gel, add the catalyst:

   50 µl 10% APS
   5 µl TEMED

15. Gently pour the stacking gel on top of the polymerised separating gel and insert the appropriate well comb, place in polythene bag and place in 37ºC incubator for up to 150 min.

16. Ensure that the stacking gel has polymerised. Remove the comb gently and pour the 1x running buffer onto solidified stacking gel. Store at 4ºC in a zip lock bag until required.

Staining of SDS-PAGE gels

1. Once the electrophoresis run is complete, disassemble the clamping frame and gel cassette sandwich. Carefully separate the 2 plates of the gel cassette.

2. Run the sharp edge of the Gel Releaser along each spacer to separate the gel from the spacer. Remove the gel by floating it off the glass plate by inverting the gel and plate under the fixative solution and gently moving the plate in the buffer until the gel separates from the plate.

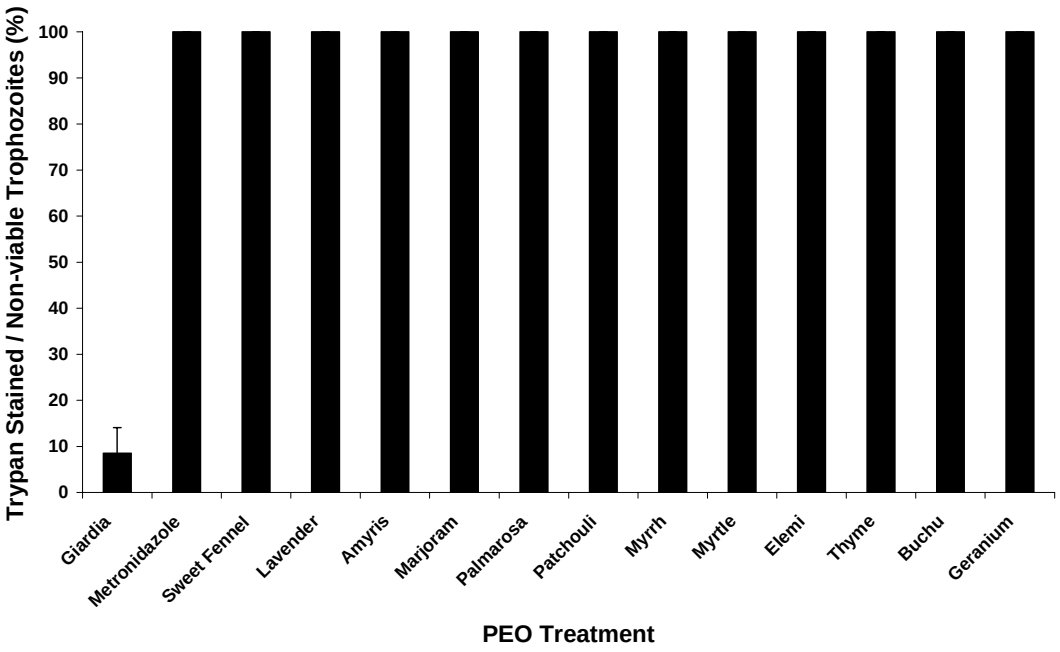
3. Rinse the Mini-Protean 3 cell electrode assembly, Clamping Frame and Mini Tank with distilled, deionised water after use.

4. Keep the gel in the fixative solution for at least 1 h then gently pour off the solution, replacing it with freshly prepared Colloidal blue G staining solution for up to 2 h. The gel may be placed in fixative solution overnight if required without any adverse effects, just as staining the gel overnight will only lead to a longer destaining period being required.

5. Pour off the Colloidal blue G staining solution and replace with acetic acid destaining solution for 30 – 60 sec and then pour off.

6. Replace with 2 washes of methanol destain solution and leave in the final wash over night. The gel can be then photographed and stored in 25% APS solution.

Appendix 6: Incubation of *G. duodenalis* trophozoites with PEOs.



G. duodenalis trophozoites were incubated for 24 h at 37°C in the presence or absence of PEOs at 0.02% or 67 μg ml^{-1} metronidazole and their viability assessed by TBMM.

Appendix 7. Determination of the effects of PEO treatment on *C. parvum* oocysts at 5°C and on the maximised *in vitro* excystation protocol.

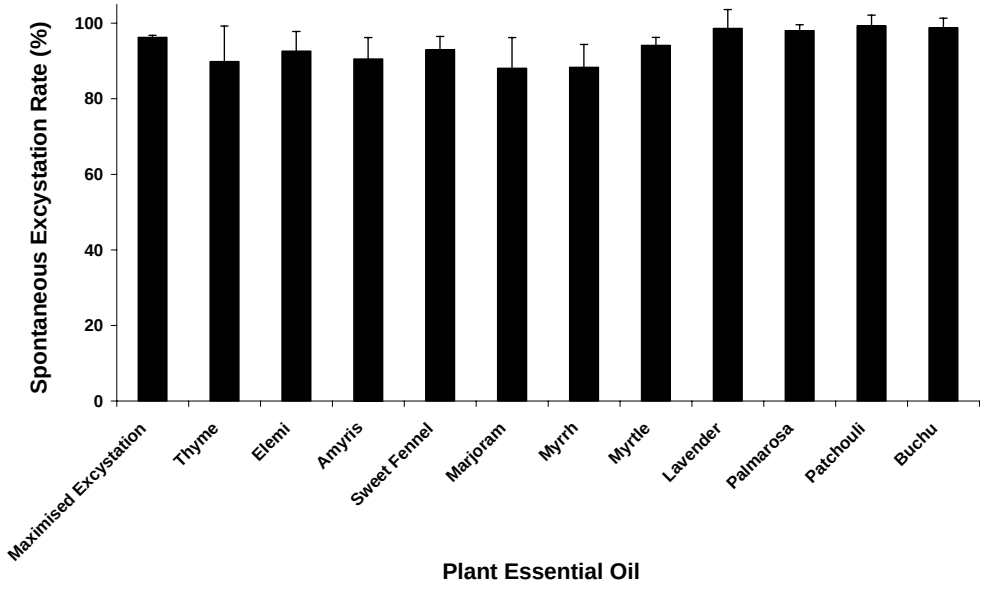


Figure 1. Determining whether PEO treatment influences maximised in vitro excystation when standard excystation triggers are used.
Oocysts were incubated for 24 h at 37ºC in the presence or absence of PEOs used at a final concentration of 0.2%. Following PEO treatment, the remaining unexcysted oocysts underwent the maximised *in vitro* excystation protocol of Robertson, Campbell and Smith (1993).

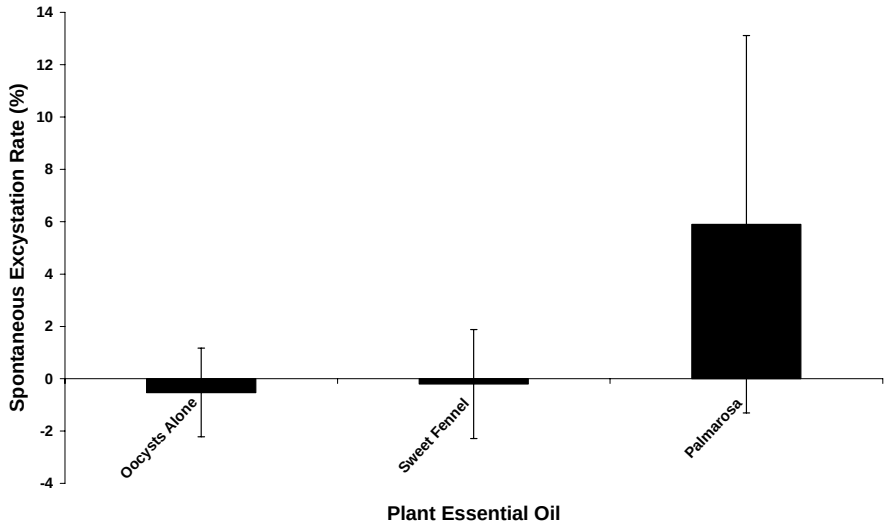


Figure 2. Determining whether PEO treatment influences spontaneous excystation rate at 5°C.
Oocysts were incubated for 24 h at 5ºC in the presence or absence of PEOs used at a final concentration of 0.2%.

Appendix 8. Agarose gel electrophoresis and PCR thermocycler programmes

50 x concentrated Tris-acetate-EDTA (TAE) buffer

242 g of TRIZMA base (Sigma-Aldrich Company Ltd, Dorset UK) was weighted and dissolved in 500ml of distilled water in a 1L beaker, to which 57.1ml of glacial acetic acid and 100ml of 0.5M EDTA, pH 8.0 (Sigma-Aldrich Company Ltd, Dorset UK) were also added. The solution was mixed on a magnetic stirrer with a magnetic stirring rod and made up to a final volume of 1L with distilled water. The expected pH is 7.5 – 8.0 and was confirmed using a pH meter (Mettler-Delta 340).

Gel loading buffer

Bromophenol blue loading buffer (bromophenol blue in 1 x TAE and 50% glycerol) was prepared by weighing 50 mg of the dye indicator bromophenol blue in a 15ml plastic universal and dissolving the powder in 9.6ml of distilled water. Ten ml of glycerol was added to this solution and 0.4ml of 50 x TAE were also added, mixed well and stored at room temperature.

Agarose gels

Agarose gels of 1.4% or 2% strength were prepared by weighing 1.2g or 2g, respectively of agarose and adding the powder to a 250ml screw top bottle containing 50ml of 1 x TAE buffer, the agarose powder remaining in the weighing boat was then rinsed with a small volume of 1 x TAE buffer and added to the bottle. The final volume was completed to either 80 or 100ml with 1 x TAE buffer. The agarose was melted thoroughly by heating in a microwave oven for 2 min. The solution was left to cool and 5µl of a 10mg ml^{-1} ethidium bromide (Sigma-Aldrich) stock solution was added to give a final concentration of 0.5µg ml^{-1}. After mixing by swirling the agarose was poured into a gel mould fitted with the appropriate comb for moulding the agarose gel wells. The gel was left to solidify at room temperature, transferred to the gel tank and submerged in 1 x TAE buffer.

The thermocycler programmes used for each PCR assay

For each pair of primers, the following cycling programme conditions (initial denaturation, annealing, elongation, final extension and total number of cycles) used is shown below. All PCR amplifications are performed in a GeneAmp® PCR Thermal Cycler, model 9700 (Applied Biosystems, Perkin-Elmer, UK).

18S.a.

PCR1:

1 cycle of 95°C for 5 min.
35 cycles of 94°C for 30 sec followed by 60°C for 30 sec followed by 72°Cfor 3 min.
1 cycle of 72°C for 10 min followed by 4°C.

PCR2:

1 cycle of 94°C for 1 min.
35 cycles of 94°C for 30 sec followed by 60°C for 30 sec followed by 72°Cfor 45 secs.
1 cycle of 72°C for 10 mins followed by 4°C.

18S.b.
PCR1:
1 cycle of 94°C for 3 min.
35 cycles of 94°C for 45 sec followed by 55°C for 45 sec followed by 72°Cfor 3 min.
1 cycle of 72°C for 10 min followed by 4°C.

PCR2:
1 cycle of 94°C for 1 min.
35 cycles of 94°C for 45 sec followed by 55°C for 45 sec followed by 72°Cfor 3 min.
1 cycle of 72°C for 7 mins followed by 4°C.

Table 1. Predicted restriction analysis pattern of the amplicon defined by primers CPB-DIAG R/F according to available GenBank sequences of the 18S rRNA gene.

Cryptosporidium species amplicon length (bp)	*Ase*I	*Dra*I	GenBank Accession No.
C. parvum 1 (438)	222, 104, 112	None	L16997
C. parvum 2 (435)	219, 104, 112	None	L16996, AF161856
C. muris (432)	320, 112	None	AF093498, AF093497
C. andersoni (431)	319, 112	None	AF093496, L19069
C. felis (455)	239, 104, 112	405, 50	AF087577
C. baileyi (428)	212, 104, 112	344, 84	L19068, AF093495
C. meleagridis (434)	171, 104, 112, 47	None	AF112574
C. serpentis (430)	318, 112	None	AF093502
C. wrairi	219, 104, 112	None	AF115378
Cryptosporidium pig	219, 104, 112	None	AF108861
Cryptosporidium desert monitor (432)	216, 108, 112	None	AF112573
Cryptosporidium mouse (439)	175, 104, 112, 48	None	AF108863
Cryptosporidium ferret (438)	174, 103, 113, 48	None	AF112572
Cryptosporidium dog (429)	213, 014, 112	None	AF112576
Cryptosporidium koala (436)	220, 104, 112	None	AF108860
Cryptosporidium kangaroo (436)	220, 104, 112	None	AF112570
Cryptosporidium monkey (436)	220, 104, 112	None	AF112569
Cryptosporidium bear (432)	216, 104, 112	None	AF247535
Cryptosporidium spp. KLJ-7 (425)	313, 112		AY324641
Cryptosporidium cervine (434)	171, 104, 112, 47	None	AF442484
Cryptosporidium QQ7 storm W	226, 112, 104	None	AF262333

Table adapted from Nichols, Campbell and Smith (2003).

PUBLICATIONS

Published papers:

Anthony, J-P, Fyfe, L., Stewart, D., McDougall, G.J., Smith, H.V. 2007 The effect of blueberry extracts on *Giardia duodenalis* viability and spontaneous excystation of *Cryptosporidium parvum* oocysts, *in vitro*. *Methods*. Vol. 42, no. 4, pp. 339-48.

Anthony, J-P, Fyfe, L., Smith, H. 2005. Plant active constituents - A resource for antiparasitic agents? *Trends in Parasitology*. Vol. 21, no. 10, pp. 462-8.

Published abstracts:

Anthony, J-P., Fyfe, L. and Smith, H.V. 2004. Inhibition of growth of *Giardia duodenalis* trophozoites by plant essential oils and other plant products. *International Giardia and Cryptosporidium Conference*, Amsterdam, Holland.

Anthony, J-P., Gold, D., Fyfe, L., Smith, H.V. 2006. Plant oils and plant stress hormones – Potentially novel drugs? *11th International Congress of Parasitology*, Glasgow, UK.

Anthony, J-P., Fyfe, L., Mitchell, S., Smith, H.V. 2006. Increased spontaneous excystation in *Cryptosporidium parvum* oocysts following treatment with plant essential oils. *11th International Congress of Parasitology*, Glasgow, UK.

Anthony, J-P., Moffat, B., Fyfe, L. Spence, G., Smith, A.L., Smith, H.V. 2006. Ancient parasites – Using the past for the future. *11th International Congress of Parasitology*, Glasgow, UK.